Investigations on the urinary excretion of adrenal cortical steroids in man

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BY

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EJNAR MUNKSGAARD · COPENHAGEN 1951

Den danske tekst af denne afhandling er af det lægevidenskabelige fakultet ved Københavns universitet antaget til offentlig at forsvares for den medicinske doktorgrad.

Fakultetet har intet ansvar for oversættelsen.

København, den 21 juni 1951

Erik Husfeldt,
h. a. dec.

PREFACE

The present thesis constitutes a collection of 5 reports on work undertaken from 1947 to 1950 during my employment in the Hormone Department of the State Serum Institute. All of the reports have been published in Acta Endocrinologica, gradually as the various stages of the work were finished

The Director of the State Serum Institute, Jeppe Ørskov, M. D., provided me with good working conditions for which I express my best thanks

The Head of the Hormone Department, Christian Hamburger, M D., has called my attention to the subject. For his never failing interest in the problems of my work and for much valuable help and advice during time I express my sincerest thanks. *The example he has set me by his excellent scientific abilities, experiences and honesty has been of invaluable inciting importance to me.*

Most of the investigations on patients were carried out at the Medical Department A, Rigshospitalet, Copenhagen, and at the Medical Department, Nørre Hospital, Copenhagen For the opportunity to take part in these investigations I am greatly indebted to Professor Knud Brøchner-Mortensen, M D, and to Professor Ejnar Jarløv, M. D.

Moreover I thank those of my colleagues who kindly have provided me with clinical material

To everybody of the laboratory staff of the Hormone Department, who gave me much useful help, I extend my sincerest thanks

To the National Danish Association against Rheumatic Diseases I am greatly indebted for grants in aid of my work

Copenhagen, June 1951

MOGENS SPRECHLER.

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In 1925 *Rogoff & Stewart* showed that adrenalectomized dogs and cats could be kept alive for a long time by the administration of AC-extract. This finding was confirmed by *Hartman et al.* (1928) and by *Swingle & Pfiffner* (1931).

In subsequent years *Grollman & Firor* (1933), *Kendall et al.* (1935) and *Wintersteiner & Pfiffner* (1935) were succesful in isolating several crystalline compounds from the AC of cattle. The substances obtained were mixtures, but were sufficiently pure to be identified as steroid hormones. The greatest difficulty encountered in the production of the extract was the removal from it of various toxic impurities, especially adrenaline without diminishing its activity. In 1936, however, *Cartland & Kuizenga* described a method for the large scale preparation of AC-extract by the extraction of beef adrenals with acetone. After a complicated purification process they ultimately produced an extract, each ml. of which represented 40 gm. of adrenal gland and contained 0.6—1.0 mg of solids. Dissolved in 0 9 per cent NaCl and 10 per cent ethanol and kept at a temperature of 4° C. it remained stable for at least a year *Kuizenga* (1943) found that extracts prepared from pig adrenals possessed much higher biological activity.

The pure crystalline AC-hormones have been isolated from such extracts. Thus *Kendall and co-workers* isolated dehydrocorticosterone and corticosterone in 1936. The latter substance was almost simultaneously isolated by *Wintersteiner & Pfiffner* and by *Reichstein*. These investigators later isolated most of the 28 different adrenal cortical steroids now known. Reichstein has been particularly successful in this work, using Girard's reagents and subsequent chromatographic analysis.

THE CHEMICAL STRUCTURE

According to *Reichstein & Shoppee* (1943) all AC-steroids hitherto isolated can be roughly divided into three groups according to the number of carbon atoms, and it is found that 24 belong to the C_{21} group, 3 to the C_{19} group and one to the C_{18} group The C_{21} group may be further subdivided by the

Acta endocrinol. 2, 70—94, 1949.

From the Hormone Department of the State Serum Institute, Copenhagen

THE CORTICOSTEROIDS, WITH SPECIAL REFERENCE TO THE URINARY EXCRETION IN NORMAL AND PATHOLOGICAL CASES A SURVEY

BY

MOGENS SPRECHLER

During the last two decades interest has been increasingly focussed on the adrenal cortex and its special hormone production, under normal as well as under pathological conditions.

As far back as 1855 *Addison* described the typical clinical picture of adrenal cortical insufficiency, and localized the site of the disease. One year later *Brown Sequard* carried out the first experimental adrenalectomies; he found that the animals died, but did not discover the real cause of this effect.

It was not until 1913 that *Biedl* established that the adrenal cortex (AC), and not the medulla, is essential for the maintenance of life, and that hormones other than adrenaline are secreted in the adrenals. As experimental animals he used a species of fish in which the two components of the adrenal are separated.

Addison's disease was first treated with adrenal cortical extract by *Osler* in 1896, but attempts to produce sufficiently pure extracts in quantities to be of use clinically were only succesful many years later.

dependent on certain features of the molecular structure, of which the following may be emphasized:

1) the 3-keto-Δ^4-group (called a α, β-unsaturated ketone group),

2) the 17-(β)-orientated side chain -CO.CH_2OH,

3) the hydroxyl or ketone group attached at C_{11}.

The two former groups are present in all the physiologically active AC steroids. A few compounds also contain 3), which seems to be essential for the effect of the steroid on carbohydrate and protein metabolism, and on the muscular activity.

Fig. 1 shows the structural formulae of active corticosteroids so far isolated from AC extract. They have, however, only a small fraction of the total activity found in the gland concentrate. After crystallisation of these steroids, the so-called amorphous fraction remains, which according to various investigations, contains up to 80 to 90 per cent of the total biological activity of the concentrate In addition to 11-dehydrocorticosterone *Lowenstein & Zwemer* (1946) have further isolated two incompletely defined substances from concentrates of AC extract. The total biological activity of these substances represent 80 per cent of the original extract. One of the substances is claimed to be a ketonic steroid having the empirical formula $C_{25}H_{34-36(?)}O_9$, and yielding ascorbic acid on mild anaerobic analysis. The substance had no specific biological effects, suggesting that is was not pure. Finally, *Hartman et al.* (1947) claim to have isolated, by chromatographic adsorption, an AC steroid which has an effect on the deposition of fat in the liver during inanition. This substance has, however, not been definitely characterized.

In addition to the above mentioned steroids, several others have been isolated which are biologically inactive and may represent conversion products or precursors of the biologically active steroids; *finally, it should not be forgotton that AC in* addition produces adrenosterone which has an androgenic effect, and also oestrone and progesterone.

number of oxygen atoms present; the following groups have been found: 8 ($C_{21}O_5$), 9 ($C_{21}O_4$), 5 ($C_{21}O_3$) and 2 ($C_{21}O_2$). The adrenal cortical steroids all contain the fundamental nuclear skeleton, i. e. the perhydrocyclopentenophenanthrene ring system in common with the sterols, bile acids and sex hormones. In the present paper only the six steroids from AC which have a hormonal activity specific for this gland and so far identified will be mentioned in detail. They are all derived from pregnane, which is a 17-ethyl derivative of ethiocholane.

A few features of the structure will be more fully discussed as they are often of great significance in the biological activity of the compound.

The methyl groups attached at C_{13} and C_{10} are always on the same side (*cis* relation) and above the ring system, which is considered to be in the plane of the paper. The bond from the carbon atom to the substituent is then shown as a solid line. It is customary to place the configuration of all other substituents in relation to the given methyl groups, so that those lying on the same side as these are said to have the (β) configuration, shown as a solid line, while those lying on the opposite side of the molecule have the (α) configuration, and are shown by a short broken line for the bond connecting the substituent to the carbon atom. Ring A (Fig. 1) is unsaturated between C_4 and C_5, this is shown by two solid lines and frequently referred to in formulae by Δ^4; the superscript refers to the carbon atom from which the double bond originates. C_{18} and C_{19} are applied to the carbon atoms in the methyl groups at C_{13} and C_{10}. The carbon atoms of the ethyl group attached at C_{17} has been called C_{20} and C_{21}

Shoppee (1947), *von Euw & Reichstein* (1947), and *Mason*, (1948), have established the position of the various substituents in the molecule, so that we now know that the side chain of C_{17} always has the (β) configuration. This also applies to the hydroxyl group at C_{11}, if such be present, while the hydroxyl group at C_{17} in the three compounds mentioned has the (α) configuration .

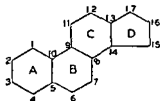
The hormonal activity of AC steroids is apparently greatly

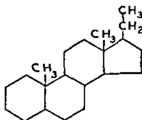
The production of the extract and the subsequent isolation of the individual substances have shown that the hormone content of AC is very small. Thus *Kendall* (1942) made quantitative determinations of 10 different steroids in an extract produced from 500 kg. of beef adrenals, and found amounts of biologically active substances ranging from 100 to 300 mg.; the amounts of desoxycorticosterone (DOC) were however negligible.

The preparation by *Steiger & Reichstein* in 1937 of DOC in vitro from the plant sterol, stigmasterol, was therefore an important advance. Later a series of the previously isolated AC steroids were synthetised including the active C_{11}-oxygenated substances with the exception of 17-hydroxycorticosterone The syntheses have been made essentially from desoxycholane acid and cholesterol. They are all partial syntheses, since it has not yet been possible to synthetise the steroid nucleus The basic material, therefore, must always be the natural substances possessing the typical »4-ring system«.

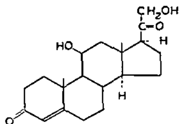
THE ADRENAL CORTICAL HORMONE

The finding of so many steroids with such different activities in the adrenal cortex has given rise to a good deal of discussion as to their true nature, with particular reference to the question. 1) whether there is one substance which is the adrenal cortical hormone proper, or 2) whether AC forms qualitatively different hormones, as in the case for the pituitary gland. The histological structure of AC is not uniform and this is in keeping with the latter view Most investigators are in favour of a dualistic view, partly because of the great difference between the effect of DOC and the C_{11}-oxygenated corticosteroids, and also because of the many different deficiency symptoms which follows adrenalectomy, and which cannot be ascribed to the absence of any one of the known steroids A few authors have claimed that there are other factors, e. g *Hartman*'s sodium factor and permeability factor.

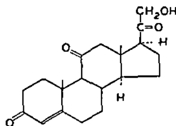




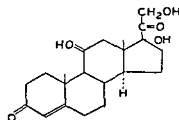
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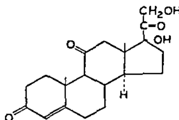
CORTICOSTERONE



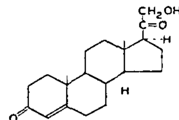
11-DEHYDROCORTICOSTERONE



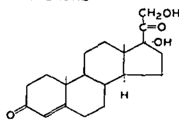
17-HYDROXYCORTICOSTERONE



11-DEHYDRO-17-HYDROXYCOR= TICOSTERONE



DESOXYCORTICOSTERONE



17-HYDROXY-DESOXYCORTI= COSTERONE

Fig 1

The structural formulae of biologically active corticosteroids so far isolated from AC extract

hormones, especially as so much about this subject is still obscure On the whole, it has been possible to attribute the changes which follow adrenalectomy to disturbances in the carbohydrate metabolism (*Boggild*, 1925; *Evans*, 1941; *Abelin*, 1946; *Jonas*, 1946), protein metabolism (*Long*, 1942) and electrolyte metabolism (*Heni*, 1947). This finding, together with the results of experiments with the individual pure crystalline steroids make it possible to group the biologically active AC steroids as follows:

I) Corticosteroids with no oxygen attached at C_{11} having a specific effect on water and electrolyte metabolism These steroids may be called mineralo-corticoids. Desoxycorticosterone and perhaps the amorphous fraction belong to this group

II) Corticosteroids with oxygen attached at C_{11} having a specific effect on protein and carbohydrate metabolism They have been named: Glucocorticoids. Corticosterone, 11-dehydrocorticosterone and their 17-hydroxy derivatives belong to this group

III) Steroids having sex hormone actions, including the androgens, the oestrogens and progesterone

This division into groups according to the effects produced is by no means absolute, but only express the most important property of any given substance. Thus glucocorticoids also have some effect on water and salt metabolism

BIOLOGICAL TESTS FOR THE DETERMINATION OF THE CORTICOSTEORIDS

These may be listed as follows:

I) a) The life-maintenance test
in the dog. *Swingle & Pfiffner*, 1932
in the rat *Kutz*, 1931.
Grollman & Firor, 1933

but, like *Kendall*'s life-maintenance factor, these can all be fitted into the dualistic theory.

Verzar, in particular, advocates the unitarian theory stressing that DOC possesses all the properties of cortin. Against this certain objections have been raised, 1) that DOC has only a slight effect, or none at all, on carbohydrate metabolism, 2) that occasionally patients with Addison's disease fail to respond completely to DOC alone, when in combination with AC-extract it is effective, and 3) that there is some doubt, whether DOC is actually present in the body. The whole question has been further complicated by recent studies which show that the secretion of hormones in the adrenal cortex is much greater than the amounts which can be isolated from the glands. This suggests that the hormone is not stored in the AC, but is being continuously synthetised from inactive precursors and immediately poured into the blood stream. This is particularly shown by the investigations of *Vogt* (1943, 1944, 1947), who determined the hormone content in the venous blood from the adrenals of dogs and, in the course of these studies, found that the 24-hour output of both adrenals in a dog weighing 10 kg. corresponded to the hormone content of 17 kg. of glands. *Vogt* calculated that this amount corresponded to about 54 mg. of 11-dehydro-17-hydroxycorticosterone or 1000 times the total daily excretion of biologically active substances in the human urine. Similar values have been found by others, e. g. by *Olson et al* (1944). At the same time it was established that cortin quickly passes from the blood stream into the tissues, even in nephrectomized and eviscerated animals. Furthermore, it was shown that there was an increased excretion of hormones from the adrenals following the injection into the animal of amounts of adrenaline similar to those levels following splanchnic stimulation (6—8 μg. per kg.). The increased excretion occurred rapidly and was of very short duration.

It would be outside the scope of this article to deal in greater detail with the mode of action of the adrenal cortical

IV) The test of the renal function in adrenalectomized dog
Pfiffner, Swingle & Vars, 1934.

The test of sodium retention in normal dog
Hartman et al., 1941.

The above test modified by use of radiosodium
Murphy & Dorfman, 1947.

The potassium excretion test in normal animals
West, 1942.

The test of semicontraction of melanophores of the carp's scales
Giroud, Santa & Martinet, 1939.

Several tests which measure the increased resistance to intoxications, poisoning and shock
Eversole, Gaunt & Kendall, 1942.
Perla & Gottesman, 1931.
Feil & Dorfman, 1945
Lewis & Page, 1946.
Elmadjian & Pincus, 1944.

Of all the tests mentioned above, only the carbohydrate tests are according to the present findings specific in the determination of a particular group of corticosteroids, viz. the glucocorticoids, which constitute perhaps the most important fraction of cortin These steroids are also the most potent in the muscular performance tests, but as some authors have also found a DOC effect in experiments with these tests, it will be necessary to retain them in a special group In the tests (I) and (IV) the activity of all the known biologically active corticosteroids are shown. The tests most commonly used have been: I d, II a, III a and b.

The cold test is based on the great sensitivity to cold of adrenalectomized rats, and the ability of the AC hormones to increase the resistance of these animals under such conditions, the prolonged survival observed after injection of the hormones being used as a criterion of the effect. It is claimed

b) The life-maintenance and growth test
Cartland & Kuizenga, 1936.
Kuizenga, 1943.

c) The growth test
Grollman, 1941.

d) The cold test
Selye & Schenker, 1938.

The cold test modified
Tyslowitz & Astwood, 1942.
Ross, 1943.

II) Carbohydrate tests (which measure the glucocorticoids):

a) The liver glycogen deposition tests
Reinecke & Kendall, 1942.
Olson et al., 1944.
Eggleston et al., 1946.
Dorfman et al, 1946 a.
Venning et al., 1946.

b) The diabetogenic test on intact rats
Ingle, 1941.

c) The diabetogenic test on partially pancreatectomized rats
Ingle, 1941.

d) The anti-insulin test
Grattan & Jensen, 1940.

III) The muscular performance tests:

a) The muscular work test of
Everse & de Fremery, 1932

b) The muscular work test of
Ingle, 1938, 1944

c) The swimming test
Gaarenstroom et al, 1937.

Fry (1940) observed that the injection of AC extract into mice brought about a very high deposition of glycogen in the liver, and on the basis of this finding, two methods were developed in 1946, one by *Venning, Kazmin & Bell,* the other by *Eggleston, Johnston & Dobriner*. In both methods adrenalectomized mice are used, the experiment starting on the 4th postoperative day. In the former method the livers of the mice are depleted of glycogen by a preceding fast; in order to obtain a greater sensitivity, glucose is administered together with the hormone. The dose of glucose should be such as not to cause any deposition of glycogen in the liver when administered alone. The total dose of the extract is given in 7 injections spread over 5½ hours One hour later the liver is removed and the glycogen content determined. The results are expressed in mg. of glycogen per 100 gm. of body weight. The assay is performed using 11-dehydro-17-hydroxycorticosterone as a standard. The biological activity of 1 μg. of this substance = 1 glycogenic unit. In the latter method the experiment is started with a certain level of glycogen in the liver, which is achieved either by injecting a certain amount of AC extract on the third postoperative day or by not removing the food until immediately before the first injection of the experiment In this method the ability of the hormone to maintain the glycogen level is determined, while in the former method the ability of the hormone to produce deposits of glycogen in the liver is determined

Both methods are considerably more sensitive (at least 50 times) than the original method described by *Reinecke & Kendall,* and it is thus possible to determine the small amounts of active substances present in urinary extracts In the experiment there is some individual variation to the response, which is, however, not so great as to prevent fairly uniform results being obtained by the use of 6 to 10 animals in each experiment. *Thayer* (1946), using rats, found an error of ± 20 per cent in the assay, and stated this is somewhat higher in experiments with mice.

A number of findings have been published on the relative

to be the most sensitive of the tests, allowing of the determination of as little as 10 μg. of corticosterone. Most investigators find, however, that animals vary greatly in sensitivity from day to day, and the individual results of each experiment show a great dispersion; a large number of animals and much extract are required, and it is advisable to use a standard dose in each assay. *Dorfman et al.* (1946 b) were able to demonstrate the effect of 0.05 ml. of AC extract. Extract from the urine of male subjects were found to be active in doses corresponding to 15 to 40 ml. of urine, but in the case of larger doses there was a steep decline in the log dose-response curve, suggesting that a toxic factor was involved which further restricts the practical application of this method. The present studies show that this method would be even less satisfactory for the routine determinations of the urinary excretion of corticosteroids. In the cold test all the corticosteroids which have a life maintaining effect on adrenalectomized animals are assayed.

The liver glycogen deposition tests are in principle based on the inability of the fasting adrenalectomized animal to form carbohydrate from protein, while at the same time the oxydation of carbohydrate in the peripheral tissue is accelerated, resulting in a depletion of the glycogen depots, especially those of the liver.

As far back as 1932 *Britton & Silvette* showed that AC extract was able to restore the blood sugar and the liver and muscle glycogen depots But it was only in 1942 that *Reinecke & Kendall* developed a method for the quantitative determination of the AC hormone, which was based on this effect. *Olson et al.* modified this method in 1944 These investigators showed that the hormone is best given in saline or in 10 per cent alcohol, and that the injection in an oily medium gave only a 50 per cent utilisation of the hormone or extract Rats were used in these methods of standardisation, which were, however, partly abandoned, because their sensitivity was not sufficiently great for the determination of the small amounts of active substances normally present in urine *Long, Katzin &*

reduced in certain pathological conditions, and that it may undergo physiological variations (*Grollman & Firor*, 1933; *Anderson & Haymaker*, 1937, 1938; *Weil & Browne*, 1939, 1940, 1944; *Dorfman et al.*, 1942, 1943; *Horwitt et al.*, 1943; *Schiller et al.*, 1943; *Shipley et al.*, 1943). An examination of the available literature shows that these pioneer studies give only few numerical data on the amounts excreted, and that the investigations have only been carried out on a few subjects.

With the adoption of the various chemical methods for the determination of these substances in the urine, however, findings in a large number of subjects have been reported during the last five years, especially in America. These included normal subjects as well as subjects with pathological conditions, and these studies suggest that the determination of the urinary excretion gives a fairly accurate indication of the adrenal cortical secretion of corticosteroids *Vogt*'s studies in particular have shown that only a small proportion of the amount actually produced is excreted in an active form, so that the amount excreted gives only a rough indication of the amount produced Furthermore it should be stressed 1) that we do not, as yet, know all the details about the structure of the biologically active corticosteroid-like substances in the form in which they occur in the urine (*Lieberman et al.*, 1947, *Mason & Sprague*, 1948), and 2) that in the determinations of the hormone by chemical methods the values obtained will often be too high as various inactive steroids are probably included.

Preparation of the urine extract

In biological tests the purity of the final extract need not be particularly high, as the assay will be unsatisfactory only if the preparation is toxic to animals. While being collected the urine should, therefore, be stored in a cool place or be treated with chloroform as preservative After completion of the collection, extraction should be done within 48 hours, otherwise the urine should be frozen, in which state it will probably keep for a very long time In the case of a high titre

activity of the various crystalline corticosteroids, e. g. by *Olson et al.* (1944), *Pabst et al.* (1947) and *Dorfman et al.* (1946 a). Most investigators find only a slight difference between the activity of corticosterone and its 11-dehydro derivative, while 11-dehydro-17-hydroxycorticosterone is two or three times so potent. The log dose-response curves of these substances are parallel, and it is interesting to note that the curve for urinary extracts has a similar slope, so that in fact they can all be used as standards. The 17-hydroxycorticosterone is the most active, but it differs somewhat from the others, and this is seen clearly by comparing the log dose-response curves, which is very steep for the latter substance. It has been suggested that the oxygenation at C_{11} is of special significance in this connection. It should be mentioned that 17-hydroxycorticosterone was also found most active in the work test (*Ingle,* 1944; *Ingle & Kuizenga,* 1945; *Pabst et al.,* 1947), and in the anti-insulin test (*Grattan & Jensen,* 1940).

When preparing AC extracts for clinical use it is important that they should contain a certain amount of the two groups of corticosteroids Unfortunately no specific test for the determination of the mineralocorticoids is at present available, and hence it is necessary to use one of the tests in group I and the sodium retention test of *Hartman et al.,* in addition it would seem desirable, as stressed by *Thayer* (1946), to test the activity of the glucocorticoids, since their content varies much more than that of the other factors.

THE URINARY EXCRETION OF CORTICOSTEROIDS

Normally the blood content of corticosteroids is so small as not to be demonstrable even with the most sensitive methods In 1931, however, *Perla & Marmorston-Gottesmann* showed that a benzene extract from urine was able to increase the resistance of the adrenalectomized rat to histamine poisoning This discovery was followed in the next few years by a large number of publications which showed that cortin is normally excreted in the urine, that its excretion may be increased or

Thompsett & Oastler (1947), however, found only slightly higher, but constant values when the partition between benzene and water was omitted. *Talbot et al.* ran the four C_{11}-oxygenated corticosteroids through all the above procedures and obtained an average of 91 per cent in the ketonic fraction. Furthermore they showed that the more oxygenated biologically active corticosteroids passed quantitatively into the water while corticosterone and its 11-dehydro derivative was removed incompletely. DOC remains entirely in the benzene. By biological assay of the ketonic fraction, *Venning et al.* found an activity approaching that which one would expect in the case of a pure crystalline glucocorticoid; but as has already been noted, the chemical determination will often give slightly higher values.

Chemical methods for the determination of the corticosteroid-like substances in urine.

1. *Fieser, Fields & Lieberman* (1944) assayed certain steroids in the urine by means of the periodic acid oxidation reaction. In 1945 *Dobriner et al* showed, however, that the method was unreliable for assaying the corticosteroids.

2. *Lowenstein, Corcoran & Page* (1946) modified the method, introducing a quantitative measurement of the formaldehyde liberated by the oxidation by which procedure 1 mol per oxygenated mol of corticosteroid is obtained Oxygenation takes place at the primary alcoholic group at C_{21}

3 *Daughaday, Jaffe & Williams* (1948) were able to use the method for the determination of the pure crystalline corticosteroids, but this method could not be applied to urinary extracts These authors therefore introduced a distillation procedure, which eliminated the unspecific coloured substances present in the urine To the destillate »chromotropic acid reagent« is added and the colour developed is compared colorimetrically with that obtained with known solutions of formaldehyde. Cortin is used as standard

a 24-hour urine specimen is sufficient, otherwise a specimen collected over 48 hours is necessary. The urine is acidified to p_H 1 with H_2SO_4, and it is then possible according to *Venning et al.* (1946) to extract an amount of biologically active material which is about twice that obtained for untreated urine. Extraction is carried out with ethylene dichloride or chloroform, ¼ vol. three times. The extract is evaporated in vacuo, and the temperature should never exceed 40—50° C, as the active substances are rather labile. The residue is taken up in chloroform, which is extracted with N/10 NaOH and water. It is then evaporated to dryness and stored in the cold (—10° C) until the day of assay.

If the extract is to be used for the chemical determination of the corticosteroids, essentially the same procedure may be used, or chloroform-ether (1:4) may be used as the means of extraction (*Heard et al.*, 1946); since, however, the chemical methods most commonly used depend on the reducing power of one or more substituents in the molecule, it is necessary to carry out the extraction very carefully, to avoid all other reducing substances which might influence the results, and only use very clean glassware and very pure reagents. Usually much smaller amounts of urine will be sufficient. The chemical assay may be carried out on the crude extract, but with this procedure there is a risk that the values obtained may be too high, one possible reason for this is the presence of non-ketonic reducing substances.

For a further purification of the extracts the procedure described by *Venning et al* (1944), and later, by *Talbot et al.* (1945) may be used:

Solution of the dry crude extract in benzene,
extraction of the benzene with water several times,
extraction of the water fraction with chloroform, removal of the chloroform by evaporation,
partition of the residue with Girard's reagent T into the ketonic and non-ketonic material,
chemical determination of the ketonic fraction.

These procedures are fairly elaborate and time consuming

In many cases a chemical determination *will undoubtedly* be sufficient, but since the values obtained may, under certain circumstances, be too high, a simultaneous biological determination would at present seem desirable. The two last mentioned chemical methods (4. and 5.) are those which have been most commonly used, but it is difficult to venture an opinion as to the *specificity of the methods before knowing* which radicals take part in the chemical reaction.

It should be pointed out *that* in the determination of the neutral 17-ketosteroids in the urine, the biologically active corticosteroid-like substances are not included.

Urinary excretion in normal subjects.

In 1946 *Venning & Kazmin* reported the results of investigations made *on a* large number of *normal subjects*, in *whom* the amount excreted was *determined biologically* by means of their liver glycogen deposition test. The results are shown in table 1.

Table 1.

The figures in brackets below the results give the average result.

	Venning et al		Heard et al.		Lowenstein et al.	Talbot et al.
	glycogenic units/24 hrs	ratio to normal	Unit DOC mg/24 hrs	ratio to normal	Unit Cortin mg/24 hrs	Unit 11-dehydrocorticosterone mg/24 hrs
Normal men	40—85 (60)	1 0	1 1—2 1 (1.53)	1 0	0 5—0 8	0 10—0.45 (0 22)
Normal women	25—65 (41)		1 0—2 0 (1 34)			
Children 2 5 year	36	0 60	0 32	0 21		
1 0 —	42	0 71	0 47	0 31		
5 5 —	53	0 93	0 70	0 46		
7 0 —	58	0 98	0 79	0 52		

It is seen that the values for women are about one-third lower than those for men Furthermore, it is interesting to note that while excretion at birth is very low, it rises fairly rapidly to reach the adult level at the 5th year, in contrast to

4. *Talbot, Saltzman, Wixom & Wolfe* (1945) used Nelson's modification of Folin & Wu's method for the determination of blood sugar to assay substances in the urine which are believed to correspond to those corticosteroids which have a ketonic or hydroxyl group at C_{11}, and which have a sugar-like or ketolic side chain and a hydroxyl group at C_{17}. The reaction is dependent on a reduction of cupric ion to a cuprous ion, which in turn reduces the arseno molybdic acid and develops a blue colour which is measured photometrically at 660 mμ. Amounts as low as 18 μg. can be measured. Corticosterone is used as a standard.
5. *Heard, Sobel & Venning* (1946) developed a method which is based on the glucose-like reducing property of the primary α-ketol side chain at C_{17} and of the unsaturated α, β-3-keto group The authors called these substances: »The neutral lipide-soluble substances of urine«. For the determination of the reduction property, Folin & Wu's phosphomolybdic acid reagent is used. The blue colour which develops is read in a photometer at 650 mμ The results are expressed in terms of mg. DOC, which is used as standard

Evaluation of methods for the determination of corticosteroids in the urine

It is difficult to decide which of the methods described gives the fullest and most reliable information about the function of the adrenal cortex which, as already mentioned, possesses a variety of functions There are good reasons for using the liver glycogen deposition test, since with this method we can measure substances of specific and high biological activity, which in all probability originate only in the adrenal cortex Besides, they constitute what is perhaps the most important fraction of the corticosteroids and are of great importance to the organism e g for protein and carbohydrate metabolism, for muscular activity and possibly other processes necessary for the maintenance of life. Moreover it would seem that they are essential for the resistance to »stress« and other external influences.

diers before and after marching, found a three-fold rise in the glucocorticoidal activity.

Pregnancy.

While no variation in excretion has been found during the menstrual cycle, *Venning* (1946), in studies on 9 pregnant women, found a moderate rise in the values in the first third of pregnancy, followed by a return to normal values. From about the 150th day the values rise again and may attain a very high level. A simultaneous determination of the 17-ketosteroids showed only a very small, or no, rise. *Tobian Jr.* (1948) assayed the urinary excretion by means of the method devised by *Lowenstein et al.* and found values in normal pregnancy twice the normal, while the values rise furthermore in case of twins, toxemia and hypertension.

THE EXCRETION IN THE URINE IN PATHOLOGICAL CONDITIONS

Hypofunction of the adrenal cortex.

This, as is well known, may be caused by a lesion in the adrenal cortex, due frequently to tuberculosis, but may also be produced by a lesion in the anterior lobe of the pituitary gland and possibly in the hypothalamus, bringing about a reduced production of the corticotrophic hormone, which results in atrophy of the adrenal cortex In Addison's disease low values are generally found, even though a few patients in good general health will show values within the normal limits. In AC-insufficiency with a hypophyseal actiology, very low values are almost always found. Hence the determination of the corticosteroids can be used to differentiate this condition from anorexia nervosa, in which the excretion is normal.

Reduced values are also frequently found in hypofunction of the thyroid gland In these cases there is a fairly good agreement with the excretion of 17-ketosteroids

the 17-ketosteroid excretion, which only reaches correspondingly high levels much later in life (*Hamburger*, 1948).

For comparison the results in a number of normal subjects tested by *Heard, Sobel & Venning* (1946) by means of their chemical method are shown in the table. Here too the average values for the two sexes show a clear difference. The figures in children are somewhat lower than those found by the biological method. It should, however, be emphasized that only a few urines from children have been assayed and, that the chemical method possibly assays other steroids in addition to the glucocorticoids.

Finally, the values obtained by *Talbot et al.* (1947) and by *Lowenstein et al.* (1946) by their respective chemical methods have been given. They found no difference between the two sexes. *Shipley et al.* (1946) studied the urine of 7 normal subjects with the cold test and obtained values corresponding to 0.5—1.8 mg. of 11-dehydrocorticosterone in 24 hours, while in twelve subjects tested with the liver glycogen test, they found amounts corresponding to 0.2—0.8 mg. of 11-dehydrocorticosterone. The cold test is not comparable to the glycogen test, since it is possible that other corticosteroids may have an effect on it The values are not directly comparable to those obtained by *Venning et al* (1946), because they used another standard, viz. 11-dehydro-17-hydroxycorticosterone. Finally it should be observed that the urine was extracted at p_H 5.5—6.5.

Diurnal fluctuations

It has recently been shown that the excretion is at its lowest level during sleep, i. e in the morning urine, and that it is higher in the forenoon than in the afternoon (*Pincus*, 1947 a; *Talbot et al.*, 1947)

Stress.

A number of studies (*Venning et al.*, 1946, *Browne et al*, 1947; *Pincus*, 1947 b) have been performed showing that the excretion rises after exertion or other »stress«. Thus *Venning et al.*, by examining the urine obtained from a group of sol-

now seem to indicate that the corticosteroid determination is of no real significance.

In table 2, an attempt has been made to list the significant values so far obtained; the scattered results on individual

Table 2

The figures in brackets above the results show the total number of subjects examined

Disease	Venning et al glyogenic units/24 hrs	Daughaday et al. (Unit Corton) mg/24 hrs (urine acidified to pH 1.7) Normal values 1.0-1.6 mg	Talbot et al (Unit 11-dehydrocorticosterone) mg/24hrs (unacidified urine) Normal values 0.10—0.44 mg
hypofunction of AC	(4) < 10	(6) 0.3-0.65	(17) 0.02—0.29
hypopituitarism	(4) < 10	(7) 0.5—0.8	(7) 0.04—0.29 6 of 7 < 0.18
anorexia nervosa	(3) 17—36		
hypothyroidism		diminished	(2) 0.07—0.13
Cushing's syndrome	(5) 137—700	(?) +++	(12) 0.6—12.0
adrenogenital syndrome	(?) norm or (+)	(?) (+)	(5) 0.24—0.57
hirsutism (simplex)	(4) 39—65		(3) 0.23—0.32

subjects found in the literature have been left out. Some investigators have subdivided the adrenogenital syndrome; this subdivision is, however, omitted from the table, which is limited to the excretion of corticosteroids.

Simple hirsutisme has been included, being undoubtedly a border line pathological condition (cf monosymptomatic forms).

Hyperfunction of the adrenal cortex.

Increased excretion of corticosteroids is found in cases in which unspecific pathological changes are present. They can all be correlated with *Selye's* theories on the adaptation syndrome. These conditions include infections, operations, burns and other serious injuries to the body (*Shipley et al.*, 1946; *Browne & Venning*, 1947). The excretion generally rises rapidly, most frequently within the first 24 hours, and the rise may persist for a very long time, not uncommonly until healing is complete. Thus, *Talbot et al.* (1947) found that with an extensive burn there were high values which persisted for as long time as 2 months. In these cases the excrétion of 17-ketosteroids is usually normal.

Among the diseases involving hyperfunction of the adrenal cortex, two distinct types may be differentiated, viz. Cushing's syndrome and the adrenogenital syndrome (*Cahill*, 1944; *Dahl-Iversen & Hojensgaard*, 1947; *Wilkins*, 1948; *Soffer*, 1948). There are, in addition many types transitional between the two and, as emphasized particularly by *Kepler*, types of the disease in which only one symptom is present The studies of *Venning et al* (1947) suggest that in Cushing's syndrome, an overproduction of the glucocorticoids is the decisive factor. They found a greatly increased excretion of these steroids, while the excretion of 17-ketosteroids was normal or slightly increased. Studies on a large number of subjects are, however, required in order to confirm these findings. In the adrenogenital syndrome the position seems to be the reverse, which is in agreement with the hypothesis that the decisive factor here is a greatly increased production of steroids with an androgenic effect; high 17-ketosteroid values but normal or slightly increased corticosteroid values are thus found (*Dobriner et al.* 1942, *Patterson et al.*, 1942, *Callow & Crooke*, 1944, *Mason & Kepler*, 1945, *Venning*, 1948; *Wilkins*, 1948)

Another problem of great practical significance in clinical medicine, is the differentiation between tumour and hyperplasia of the adrenal cortex The studies carried out up til

now seem to indicate that the corticosteroid determination is of no real *significance*.

In table 2, an attempt has been made to list the significant values so far obtained; the scattered results on *individual*

Table 2

The figures in brackets above the results show the total number of subjects examined

Disease	Venning et al glyogenic units/24 hrs	Daughaday et al. (Unit Cortin) mg/24 hrs (urine acidified to pH 1 7) Normal values 1 0 - 1 6 mg	Talbot et al (Unit 11-dehydrocorticosterone) mg/24hrs (unacidified urine) Normal values 0 10 - 0 41 mg
hypofunction of AC	(4) < 10	(6) 0 3 – 0 65	(17) 0 02 – 0 29
hypopituitarism	(4) < 10	(?) 0 5 – 0 8	(7) 0 01 – 0 29 *6 of 7* < 0 18
anorexia nervosa	(3) 17 – 35		
hypothyroidism		diminished	(2) 0 07 – 0 13
Cushing's *syndrome*	(5) 137 – 700	(?) +++	(12) 0 6 – 12 0
adrenogenital syndrome	(7) norm or (+)	(?) (+)	(5) 0 21 – 0 57
hirsutism (simplex)	(4) 30 – 65		(3) 0 23 – 0 32

subjects found in the literature have been left out Some investigators have subdivided the adrenogenital syndrome; this *subdivision is, however,* omitted from the table, which is limited to the excretion of corticosteroids.

Simple hirsutisme has been included, being undoubtedly a border line pathological condition (cf monosymptomatic forms).

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INVESTIGATIONS ON THE CHEMICAL DETERMINATION OF CORTICOIDS IN URINE

BY

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Numerous steroids have been isolated from human urine in recent years and their structure is now to a great extent established. Some of them are derived from the gonads but most of them are probably metabolites of the steroid hormones elaborated by the adrenal cortex. These include the biologically active corticoids (glucocorticoids with a specific effect on protein and carbohydrate metabolism, and mineralocorticoids with a specific effect on water and electrolyte metabolism), androgens, oestrogens and progesterone. In addition to these, however, there are a large number of biologically inactive steroids closely related to the above mentioned groups.

Up to quite recently the chemical nature of the biologically active substances in the urine, especially the so-called glucocorticoids, was unknown. In 1948 *Mason et al.*, however, isolated large amounts of 17-hydroxycorticosterone in the urine from a patient suffering from Cushing's syndrome and recently *Schneider* (1950) isolated 55.3 mg. 17-hydroxy-11-dehydrocorticosterone (cortisone) from 1000 litres of normal male urine The excretion per 24 hours was calculated to be of the order of 82 μg

Various biological as well as chemical methods have been proposed for the quantitative determination of the urinary corticoids (reviewed by *Sprechler*, 1949). In practice the most

useful biological tests are those based on the capacity of the glucocorticoids to cause glycogen deposition in the liver of the fasting adrenalectomized animal. In this respect the method of *Venning et al.* (1946) appears to be one of the most sensitive.

Two types of chemical methods have been worked out. One of these is based on the reducing property of the α-ketol side chain at C_{17} for the cupric ion (*Talbot et al.*, 1945) and for phosphomolybic acid (*Heard & Sobel*, 1946). The last-mentioned reagent also reacts with the α, β-unsaturated keto group at C_3. These two groupings are always present in the molecule of the typical biologically active corticoids. The other type of method (*Lowenstein et al.*, 1946, *Daughaday et al.*, 1948) depends on the measurement of the formaldehyde released on periodic oxidation of the primary α-ketol grouping at C_{17}. In addition, however, formaldehyde can also be produced from steroids hydroxylated at C_{20}.

OWN INVESTIGATIONS

The aim of the present study was to re-examine the existing chemical methods for the determination of urinary corticoids.

At first the most simple method devised by *Heard, Sobel & Venning* (1946) was explored. More than 500 analyses were carried out in the course of one year. Several of these were performed in duplicate, and after some practice a good agreement was obtained between the two assays In several cases, however, different coloured substances passed from the urine into the lipoid solvent and interfered with the blue colour in the colorimetric assay. It has not been possible to obtain a correction for this unspecific colour. Furthermore very large day to day variations were found in the results of analyses carried out on hospital specimens from patients kept under uniform conditions in medical departments This must have been due to unspecific reducing substances and was not found when using a method which will be described later *Heard, Sobel & Venning* (1946) suggested in an addendum to their paper that

special precautions must be taken during the collection of urine in order to avoid contamination. This is impracticable if a large number of analyses have to be performed by routine procedure and as it was not possible to obtain reliable estimations, especially for routine purposes the method was abandoned.

Subsequently the method of *Talbot et al.* (1945) was investigated. In this troublesome and time-consuming method a thorough fractionation of the crude urinary extract is necessary before the final colorimetric assay. In all the experiments, however, insuperable difficulties were encountered in the final chemical procedure, because of precipitation in, and cloudiness of, the reaction mixture. Some investigators have suggested that these insoluble substances should be extracted by shaking with ether, but this was found quite unsuitable as it gave unreliable results in control assays. *Heard & Sobel* (1946) observed the same difficulties and ascribed them to extreme insolubility of most steroids in the aqueous alkaline copper solution. The same investigators also stated that a benzene-water partition is not specific for the glucocorticoids as suggested by *Talbot et al*, who found that 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone were recovered quantitatively from the aqueous phase, while corticosterone and 11-dehydrocorticosterone remain mostly in the benzene (57 and 71 per cent respectively). *Heard* and *Sobel* also stated that an 11-desoxy-compound, $\triangle^4$-pregnene-17, 20, 21-triol-3-one, is recovered in water.

The method based on the formaldehyde liberating power on the periodic oxidation could not be examined here as we were unable to obtain the necessary reagents The results mentioned above led us to base the chemical assay on the principles described by *Heard & Sobel* but in order to get a higher specificity in the determination, we have performed a further fractionation of the total neutral urinary extract as proposed by *Talbot et al* Girard's reagent T has been used for this purpose The various steps in the procedure have been critically studied, and the results of these experiments are given below

REAGENTS

Sodium hydroxide, sulphuric acid, glacial acetic acid and *anhydrous sodium sulphate* are all analytical reagents.

Chloroform (Ph. Dan.) is freshly redistilled.

Girard's reagent T (Ciba) must be kept in a dessicator or in ampoules.

Phosphomolybdic acid solution (*Folin & Wu*, 1920): 35 gm. molybdic acid and 5 gm. sodium tungstate are dissolved in 200 ml. 10 per cent sodium hydroxide and 200 ml. of distilled water are added. In order to remove the ammonia it is boiled vigorously for 20—40 minutes. After cooling and dilution to 350 ml. with distilled water 125 ml. of concentrated phosphoric acid are added Finally the solution is diluted to 500 ml. with distilled water.

Phosphomolybdic acid reagent (*Heard & Sobel*, 1946): Immediately before use, the reagent is made by mixing equal parts of the above-mentioned phosphomolybdic acid solution and glacial acetic acid.

Molybdic acid is prepared as follows: A thin layer of ammonium molybdate is placed in a pyrex glass tube of high melting point, through which a stream of oxygen is passed. On intensive heating the ammonium molybdate is transformed into needles and into leaves of molybdic trioxide (Mo_4O_9). By the use of this technique it has been possible to obtain a reagent that is stable for a long time when kept in the cold and in the dark.

Several reducing substances react with the phosphomolybdic acid and it is, therefore, essential to take every precaution when performing the analyses The lubricants used must be controlled The glass-ware must be cleaned by washing with water, alcohol and ether. All distillations must be carried out in all-glass apparatus Last but not least, the inside of the glass-ware and the tips of funnels etc should not be touched In order to control the specificity of the analysis itself, blank determinations have been performed

THE URINE

A 24-hour urine specimen is collected without preservative and kept in the cold up to the period of extraction, which should be performed as soon as possible. If this cannot be done, the urine should be kept frozen in a refrigerator.

If a normal content of corticoids is expected, $^{1}/_{10}$ of the 24-hour specimen is used for the analysis If this amount is less than 80 ml. the urine is diluted to this volume with distilled water, as it is an advantage to work with not too small an amount of urine

ACIDIFICATION

Talbot et al. (1945) extracted unacidified urine but *Heard, Sobel & Venning* (1946) and others obtained considerably higher yields from acidified urine assayed chemically. This has been confirmed by *Venning et al* (1946) who used a biological test for assaying the glucocorticoids. These investigations show that some of the urinary corticoids must be present in a conjugated form

In order to find *the optimal pH value* several experiments at different pH levels have been performed on various urines, the extraction being performed immediately after acidification. In some instances the total neutral extract has been divided into two equal parts One of these is treated with Girard's reagent as described later, the other is partitioned between benzene and water, using the method of *Talbot et al.* (1945), after which the reducing power of the total water-soluble steroids is determined.

In both cases the yield was 75—100 per cent higher at pH 1 as compared with the result obtained by the extraction of unacidified urine (Fig 1). The maximum liberation of corticoids from their conjugates seems to take place at a pH of between 2 and 1

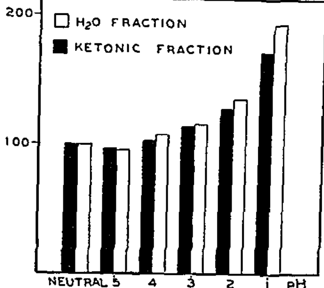


Fig 1

The effect of acidification of the urine on the extraction of corticoids. The columns represent the average result of experiments carried out on 5 different urines

Aliquots of 24-hour from 6 women and 5 men were also adjusted to pH 1 and pH 2. From the results obtained by extraction at pH 1 and pH 2, respectively, the ratio of pH 1 : pH 2 has been calculated (Table 1). On an average these ratios appear to correspond closely with the results illustrated in Fig. 1. It is of interest that the ratio is found to be higher for men than for women. *Heard, Sobel & Venning* (1946) observed a similar sex difference with regard to the absolute excretion values.

It must be assumed that the acidification causes a mild hydrolysis. It is, therefore, of interest to determine *the effect of time* on this hydrolysis. Several analyses were performed in the following manner: Large amounts of urine were adjusted

Table I

Ketonic corticoids, mg. per 24 hours					
women			men		
pH 1	pH 2	$\frac{pH\ 1}{pH\ 2}$	pH 1	pH 2	$\frac{pH\ 1}{pH\ 2}$
0 60	0 44	1 36	1.40	0 75	1 87
0 44	0 30	1 46	1 03	0 66	1 56
0 76	0 63	1 21	0 90	0.56	1 61
1.03	0 83	1 29	1.33	0 95	1 40
0 76	0 53	1 43	0 66	0 41	1 61
0 80	0 53	1.51			
0 73	0.54	1 35	1 06	0 67	1 58

The urinary excretion of corticoids in 6 women and 5 men
The extraction was carried out at pH 1 and pH 2

to pH 1 and then divided into a number of equal portions. Extraction was carried out immediately after the acidification and then hourly for the following 8 hours, and finally after standing for 24 hours at room temperature.

Such large variations from one urine to another were found that it was impossible to give any general rules for the optimum time of hydrolysis. After 5—6 hours, however, increasing amounts of extractable reducing ketonic corticoids were found in most cases The highest values appear to be reached after 24 hours' hydrolysis, but biological determinations performed simultaneously showed lower values for the content of glucorticoids, indicating a destruction or a conversion of the biologically active corticoids into inactive products

In order to eliminate this destructive effect of the acid upon the labile part of the corticoids, it was decided to carry out the extraction immediately after the acidification to pH 1 (measured with a glass electrode) and using 40 vol. per cent H_2SO_4, though we realised that these were not the optimal conditions.

EXTRACTION

Heard, Sobel & Venning (1946) used a chloroform-ether mixture 1 : 4. *Talbot et al.* (1945), however, found ether less suitable as the partition between this solvent and water for several steroids is 1 : 1. They found that a small volume of chloroform extracted practically all the corticoids.

Experiments have shown that even the most purified ether available to us (Ph. Dan.) contains some unspecific reducing substances, which are not completely removed by redistillation. Redistilled chloroform, however, gives satisfactory results, as shown by several control analyses (»water blanks«). It is of interest that, when working with *Heard & Sobel*'s method, we always found much higher values for the urinary content of corticoids when chloroform was used as the extraction solvent.

Several assays (Table 2), performed with different volumes of chloroform, show that satisfactory recoveries can be obtained by shaking the urine vigorously three times with 25 per cent (by volume) of chloroform in a separating funnel. The emulsions, which are practically always formed, are broken down by centrifugation. If the urine contains large quantities of pus and albumin it is necessary to centrifuge it before the extraction. The chloroform extracts are combined in the separating funnel and washed by shaking three times *with 10 ml. of 0.1 N NaOH and then three times with 10 ml.* of distilled water. Each washing is extracted back with 5 ml. of chloroform and these are added to the original chloroform extract before going on with the next washing. This re-extraction is especially necessary as the NaOH will otherwise cause a small loss each time Several yellow-brown coloured substances and phenols are removed by shaking with NaOH These substances can interfere unspecifically with the colorimetric assay.

The chloroform extract is dried with small amounts of anhydrous sodium sulphate, filtered and evaporated to dryness in an all-glass vacuum distillation apparatus, the temperature of the water-bath being kept at 45° C.

Table 2

Chloroform per cent (by volume)	number of extractions	Amount of reducing ketonic corticoids in urine number			
		I	II	III	IV
		µg	µg	µg	µg
25	1	54	26	21	226
25	2	65	30	26	278
25	3	58	34	29	376
25	4	59	35	33	388
25	5	63	35	33	
50	2	59	25	31	
Chloroform per cent (by volume)	**successive extraction**	**urine number V**	**ketonic fraction in 80 ml of water (83 µg)**	**Cortin (§) 0.5 ml in 80 ml of water**	
		µg	µg	µg	
50	I	16	77	84	
50	II	12	3	11	
50	III	1	0	2	
100	I	25			
100	II	1			
100	III	2			

The effect of amount of chloroform and number of shakings on the extraction of reducing ketonic corticoids from urine and of ketonic corticoids and cortin added to water

§) Cortine (Organon) = adrenal cortical extract

The analysis can be interrupted at this stage and the neutral dry *residue can be kept for a long time if stored in a re*frigerator.

The recovery of desoxycorticosterone and 17-hydroxy-11-dehydrocorticosterone added to normal male urine and carried *through the procedure described above is shown in Table 3.*

GIRARD'S SEPARATION

In 1936 *Girard & Sandulesco* introduced the ketone-reagent, trimethyl acethydrazide ammonium chloride (Girard reagent

Table 3

Urine (pH 1)		DOC added	Cp. E added	Reducing value (DOC Standard)	Recovery
	ml	µg	µg	µg	µg
A	80	0		209	0
	80	50		260	51
	80	100		314	105
	80		100	281	97*
	80		200	358	202*
			Cp. E. ac.		
B	80			580	
	80	100		688	108
	80		100	631	100×
	80		200	676	188×

*Recovery of desoxycorticosterone, Cp. E and Cp. E acetate from normal urine, *, × expressed as the Cp. E and Cp. E acetate equivalent respectively The determination is carried out on the total neutral fraction.*

T) as a means of separating ketonic from non-ketonic steroids. This process, which has been of immense value for investigating the nature of steroid substances, is based upon the fact that, *under certain conditions,* ketonic steroids form water-soluble hydrazone derivatives with the reagent. These compounds are insoluble in organic lipoid solvents, and by extracting the mixture with ether or chloroform it is possible to remove the non-ketonic substances. The ketones can be liberated again from the hydrazone complex by acid hydrolysis and extracted afterwards with organic lipoid solvents.

Girard & Sandulesco (1936) dissolve the urinary extract in absolute alcohol. 10 per cent glacial acetic acid and 5—10 per cent reagent T are added The mixture is heated on a boiling water bath for 30—60 minutes, and then cooled and transferred with ice-cold water to a separating funnel. Sufficient alkali is added to neutralize 90 per cent of the acetic acid and the non-ketonic steroids are immediately extracted with ether The remaining watery solution is acidified to a 0.5 N concentration with HCl or H_2SO_4 and after standing for one hour

at room temperature, the ketonic steroids are extracted with ether.

Reichstein (1936) introduced some modifications, especially a fractional extraction of the ketonic steroids at different pH values, and used this method for the separation and purification of steroids from adrenal cortical extracts. He observed that the saturated steroids are easily liberated from the hydrazone complex by mild acid hydrolysis with weak acids, whilst unsaturated steroids, including the 3-α,β-unsaturated compounds, require strong acids and a longer time for hydrolysis.

Pincus & Pearlman (1941) worked out a micro-method for the separation of the 17-ketosteroids in small amounts of urine (24 hours samples). In this modification, the reaction is performed with the dry steroid to which 0.5 ml. glacial acetic acid and 100 mg reagent T are added.

Talbot et al (1945) used a similar method for fractionating the water-soluble corticoids obtained after the benzene-water partitioning of the crude urine extract

Several other authors have used the original Girard method or modifications thereof (*Talbot et al.*, 1940, *Venning et al*, 1944, *Schiller et al*, 1945, *Dobriner et al*, 1948). The main differences between the methods are in ratio of reagent to steroid, the temperature used during the process, the amounts of alkali and water, the time of hydrolysis of the hydrazones and the extraction procedures.

When the Girard process was adopted for a further fractionation of the corticoids for routine analyses, it obviously became necessary to determine the optimal conditions for the various steps in the process

Amount of water and solvent. It was found that the process could be satisfactorily performed with small amounts (25 ml) of water and lipoid solvents provided that the amount of steroid did not exceed 10 mg and this micro-method has been used.

Dry steroid versus alcoholic solution Several experiments were carried out with 10 mg dehydroisoandrosterone (DHA) in a dry form or dissolved in 2 ml. alcohol No significant dif-

ferences in the recoveries were obtained. We have therefore adopted the dry method, and the steroid is dissolved in only 0.8 ml. glacial acetic acid.

Amount of Girard reagent T: Most of the experiments were carried out with 10 mg. DHA and the quantity of reagent varied from 10 mg. to 200 mg. With 20 mg. of reagent or more the recoveries were maximal, i. e. about 95 per cent. In experiments with 111 µg cortisone the amounts of reagent were 12.5, 25, 50 and 100 mg. respectively, and the smallest quantity was found to be sufficient (97 per cent recovery in the ketonic fraction). In order to be certain that a sufficient amount of reagent is used when assaying urines with various and sometimes high corticoid contents, *we use 25—40 mg. of reagent* in the routine analyses.

Conditions for the hydrazone formation. Boiling versus room temperature· Girard & Sandulesco (1936) used boiling for 30—60 minutes; *Talbot et al* (1940) heated the reaction mixture for 10 minutes on a boiling water-bath; *Pincus & Pearlman* (1941) warmed the mixture for 20 minutes at 90—100° C, while *Venning et al* (1944) left the reaction mixture at room temperature for about 15 hours. Some of our own experiments with DHA, urinary extract of 17-ketosteroids, cortisone, DOC and a urinary extract of corticoids are summarized in Table 4

As is evident from Table 4, almost complete recoveries are obtained after heating for 5 minutes or more on a boiling water-bath, or when the reaction mixture is left at room temperature overnight. The last-mentioned technique probably gives better and more consistent results. In the routine work it is an advantage if the analytical procedure can be interrupted at this stage and continued next morning

Amount of alkali used for neutralization of the acetic acid Most authors have adopted the original technique of *Girard & Sandulesco* and added sufficient alkali to the reaction mixture to neutralize 90 per cent of the acid. We have carried out several experiments with various amounts of NaOH and found that as soon as the pH is on the alkaline side, the recoveries were maximal, i e about 95 per cent. Before neutralization,

Table 4

Boiling water bath in minutes	Amount of corticoids recovered in ketonic fraction				
	DHA (10 mg.)	Cortisone (111 μg)	DOC (100 μg)	urinary extracts: 17-ketosteroids (10 mg.)	urinary extracts: corticoids (L. 15)
	mg	μg	μg	mg	mg. per litre
2	7.65 (3)	103 (2)	91 (2)		0.68 (4)
5	9.67 (6)	105 (2)	90 (2)	10.0 (3)	0.67 (2)
10	9.60 (3)	100 (2)		9.5 (4)	
15	9.34 (3)				
30	9.20 (9)			9.76 (2)	
Left at room temperature overnight	10.3 (2)	104 (5)	92 (6)	9.76 (2)	0.72 (5)

The effect of heating on boiling water bath, or of standing overnight at room temperature, on the hydrazone formation in the Girard process as measured by recoveries in the ketonic fraction. In addition to the results the figures in brackets also show the number of analyses *performed*

the test-flask is cooled and the reaction mixture transferred to a separating funnel with 25 ml. of ice-cold water. It was, however, found that more consistent results can conveniently be obtained if the acid mixture is transferred with a dilute alkaline solution containing the required amount of NaOH. In the routine analyses this has been performed by the use of 25 ml 0.55 N NaOH at 0° C From the neutralized mixture the non-ketonic substances are then extracted by shaking 3 times with 25 ml. of ice-cold chloroform

Amount of acid necessary for the liberation of ketones from the hydrazone complex. The amount of acid necessary depends on the aim of the process When the Girard process is used as a means of identifying steroids, successive extractions at different pH values are performed In clinical assays, in which a determination of the total amount of ketones is desired the extraction is always carried out at only one pH value *Girard & Sandulesco* added HCl or H_2SO_4 in such amounts that a 0.5 N concentration was obtained Different amounts of these

two acids have been used by other investigators. Our own experimental data are given in Table 5. From the table it can be seen that consistent results can be obtained over a wide range of acid concentrations. Immediately after acidification 25 ml. chloroform are always added.

Table 5

Amount of 40 vol per cent H_2SO_4 added for the acidification	DOC added	DOC recovered	Cortisone Acetate added	Cortisone Acetate recovered	Urinary extract L-52
ml.	μg	μg	μg	μg	mg. per litre
1.0	100	89			0.30
1.5	100	92	200	200	0.44
2.0	100	93	200	184	0.44
2.5	100	95	200	189	0.44
3.0	100	93	200	181	0.39
3.5	100	98	200	184	0.42

The effect of the amount of added H_2SO_4 on the recovery of steroids in the ketonic fraction of the Girard process

Some of the biologically active corticoids are sensitive to strong acids and *Heard, Sobel & Venning* (1946) found a marked loss in the reducing power of DOC and urinary corticoids after standing for 1 hour at room temperature in a medium containing 2 volumes per cent of sulphuric acid. In our experiments no significant loss was found even at the pH value of 0.4. It is our impression, however, that the free crystalline compounds and urinary corticoids, when dissolved in water, are very easily extracted by means of chloroform and this in all probability is explained by the fact that the liberated steroids rapidly pass from the acid aqueous phase to the lipoid solvent.

In order to ensure a quantitative hydrolysis of the hydrazone complexes, we finally decided to use 2 ml. 40 vol. per cent H_2SO_4 which brings the pH value to just below 1.0. With this amount of acid the recoveries have been satisfactory in all cases.

After standing at room temperature for two hours, the ketonic fraction is extracted by shaking with the chloroform already present, and then further extracted twice with 25 ml. of chloroform.

The combined chloroform extracts are washed once with 10 ml. 0.1 N NaOH and three times with 10 ml. distilled water. Each washing is extracted back with 5 ml chloroform as described above. After drying with anhydrous sodium sulphate and filtering, the chloroform extract is evaporated to dryness in an all-glass vacuum distillation apparatus at 45° C The dry residue is then carefully transferred to a test-tube three times with 1 ml. of chloroform. After evaporation of the chloroform the test-tube can be stored in a refrigerator

THE COLORIMETRIC ASSAY

To the dry residue two ml. of the phosphomolybdic acid reagent are added, the test-tube is closed with a cork wrapped with aluminium-foil and placed in a boiling water-bath for exactly 60 minutes as described by *Heard & Sobel* (1946) Immediately after cooling to room temperature, 8 ml of the reagent are added and the mixture is transferred to a cuvette (internal diameter 16 mm). The optical density is measured in a Coleman photometer (model 14) at 700 mμ using a red filter (PC-5) The adjustment to zero optical density is performed with a reagent blank which is carried through the procedure mentioned above. The absorption curve for the molybdenum blue shows no maximum but a plateau from 685 mμ to 800 mμ.

Because of the difficulty in obtaining sufficient amounts of molybdic acid it has been necessary in most cases to use 50 per cent acetic acid for the dilution of the reagent mixture after the colour has been developed. *Heard & Sobel* (1946) found that this procedure can be used if the reading is per-

formed within 3 minutes, after which time the optical density decreases. We have been able to confirm this observation. It should be pointed out that the result is affected by the temperature at which the colour is developed. It is therefore very important to maintain uniform conditions for boiling. An electric hot plate or a steamboiler can be used.

Desoxycorticosterone is used as the standard of reference. A linear standard curve is found when the doses of DOC (e. g. 50, 100, 150, 200 µg) are plotted against the values for the optical density. From this the reducing equivalent of the urinary extract can be determined.

It has been necessary to control the standard curve for each new batch of reagent as it has been impossible to obtain uniform batches of molybdic acid.

AMOUNT OF CORTICOIDS IN THE URINE

It is not, as yet, possible to give the limits of the urinary excretion in normal human subjects but assays carried out on the urines from a small number of healthy subjects indicate that the values are of the order of 0 40 and 1.0 mg. per 24 hours These values correspond rather closely with those obtained by estimating the formaldehyde liberating corticoids. The following results are mentioned in the literature: *Heard, Sobel & Venning* (1946) 1 0—2 0, *Lowenstein et al.* (1946)· 0 5—0 8, *Talbot et al* (1947). 0.10—0.44, *Corcoran & Page* (1948). average 1.0 mg All these values represent the excretion per 24 hours in normal adults

A patient suffering from Addison's disease excreted 0 20 mg. per 24 hours in a state of crisis suggesting that an extra-adrenal source may produce some part of the reacting substances On the same day the 17-ketosteroid excretion was found to be 0.5 mg so that the presence of small remnants of functioning adrenal cortical tissue cannot be excluded

EVALUATION OF THE METHOD

As the chemical test offers the advantages of speed and comparatively *low variability (duplicate analyses are shown* in Fig. 2), it is preferable to the troublesome and time con-

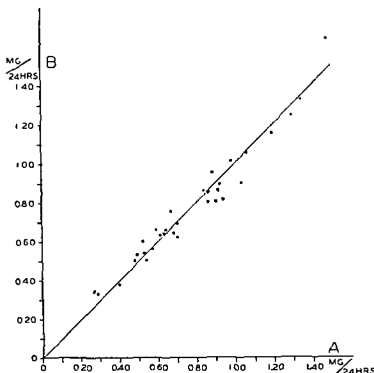


Fig 2
33 duplicate analyses plotted against each other.

suming procedure involved in biological tests. The available literature, however, reveals only a few attempts to correlate the values for the excretion of corticoids as determined by means of the chemical and biological methods, respectively, and hence some experiments were carried out on this question

Shortly after the first reports from U. S A. on the successful treatment of chronic rheumatoid arthritis with ACTH had reached Denmark, similar investigations were undertaken

here (*Brochner-Mortensen et al.*, 1949). Several hundred 24-hour urines from ACTH treated patients have been analyzed for their corticoid content. We thus had ample opportunities for examining, firstly the agreement between the chemical and biological determinations, and secondly the correlation between the adrenal cortical function and the amounts of corticoids excreted.

24-hour specimens of urine were extracted in the usual manner and the total neutral fraction obtained was divided in aliquots for further processing, viz. chemical determination as described above and biological determination of the glucocorticoids by the method of *Venning et al.* (1946). Furthermore, in two cases a benzene-water partitioning was carried out, and the reducing substances obtained in the water fraction were colorimetrically determined as described above. Fig 3 illustrates the data for the chemical assays in the two cases. In case J D there is a very close agreement of the absolute output values and in both cases there is a remarkably consistent correlation with regard to the relative output changes Some time after the ACTH treatment, testosterone propionate was administered to one of the patients During this period there was a marked increase in the excretion of 17-ketosteroids as determined by the micro-method of *Hamburger* (1948), but as can be seen, there was no effect on the excretion of corticoids, clearly indicating that the 17-ketosteroids do not interfere with the colorimetric assay of the corticoids From these findings it is evident that no advantages is to be gained from a benzene-water partitioning of the urinary corticoids.

In Fig 4 the results are shown of parallel chemical and biological determinations performed on urines from three patients, i e the two mentioned above and a third patient The excretion curves for the biological determinations are more irregular during the ACTH treatment because of the greater variability in the bioassay. In all cases it is obvious that there is rather good agreement between the values obtained from day to day by the two methods The percentage increase over the pre-treatment level is, however, more pronounced in the

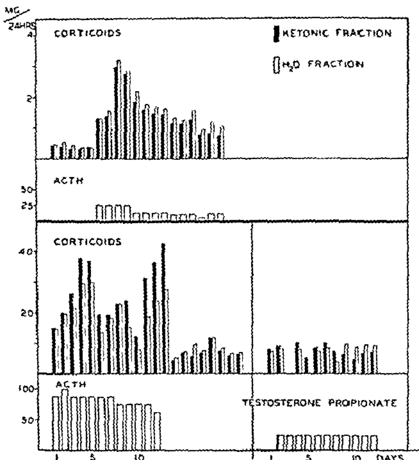


Fig 3
The excretion of reducing ketonic corticoids and total water-soluble corticoids in two cases treated with ACTH (Case J D. above, and case L R below)

bioassay than in the chemical test. As an example, case A. R. may be considered In this patient a hundred percent increase in the reducing corticoids was found while the increased excretion of biogenically active glucocorticoids amounted to about 300 per cent.

This could be best explained on the assumption that the

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litative differences in activity, when assaying several adrenal cortical extracts and the crystalline glucocorticoids The problem will be further discussed in a later publication.

From several analyses carried out with urines from different patients and from normal human subjects it is clear that the absolute values for the diurnal excretion of reducing ketonic corticoids are consistently 8—10 times larger than those obtained by a simultaneous biological determination of the glucocorticoids. In the chemical assay, however, biologically active as well as biologically inactive metabolites are included and this fact must surely explain the difference observed. Very similar findings have been obtained in the chemical determination of 17-ketosteroids, as compared with the biological assay of the androgenic substances.

It can be concluded that all the neutral ketonic corticoids, as estimated by their reducing power, are metabolites closely related to the urinary biologically active glucocorticoids which are probably included among them Furthermore it should be stressed that the chemical as well as the biological determinations reflect the cortical function in a similar manner

SUMMARY

After a thorough experimental examination of the method of *Heard, Sobel & Venning* and of *Talbot et al.* for the determination of the urinary corticoids they were both abandoned. A method based on a combination of the principles used in the two methods is described. It is shown, however, that no advantage is gained by a benzene-water partition of the corticoids. An investigation on the different steps used in the analysis has been carried out in order to establish the optimal conditions.

The technical details in the procedure are outlined in the diagram below.

By means of simultaneous chemical and biological determinations on urines from ACTH treated patients, the relation between the reducing ketonic corticoids and the biological-

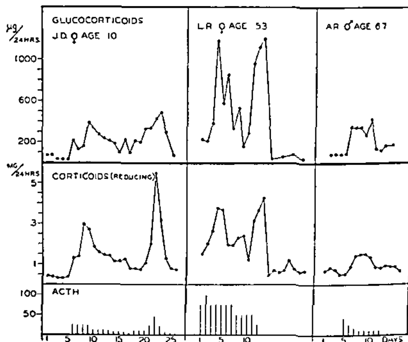


Fig 4
The excretion of reducing ketonic corticoids and biologically active glucocorticoids in three cases treated with ACTH.

catabolism of the corticosteroids cannot keep pace with the greatly accelerated elaboration of these substances which occurs during ACTH-stimulation of the adrenal cortex. This results in a high titer of these biologically active corticosteroids in the blood, which in turn is reflected in a relatively higher excretion in the urine

In addition, however, the possibility of a qualitative alteration in the composition of the urinary corticoids during the ACTH treatment must be kept in mind. If the regression line for the urinary glucocorticoids under these conditions acquires a different slope, as compared with the standard curve for cortisone, this should be considered as a part of the discrepancy observed In this connection it is of interest to note that *Olson et al.* (1944), using a similar method, found similar qua-

ly active so-called glucocorticoids is shown The agreement between the data is discussed. Both sets of results appear to reflect the adrenal cortical function in a similar manner.

ACKNOWLEDGEMENT

I am greatly indebted to Dr. *J. Heer* and Dr. *A Wettstein*, Ciba, Basle, for the preparation of the free crystalline 17-hydroxy-11-dehydrocorticosterone, to *Ciba Limited*, Basle, for supplying me with desoxycorticosterone, and to Dr. *Frederik Paulsen*, Organon, Stockholm, for a Cortine preparation

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Adjust 1/10 of 24-hr urine to pH 1 with 40 vol. per cent H_2SO_4

extract 3 times with re distilled chloroform 25 per cent by volume

combine the chloroform extracts and wash 3 times by shaking with 10 ml. 0.1 N NaOH and 3 times with 10 ml. of water. Each washing is re-extracted with small amounts of chloroform

dry the extract with Na_2SO_4, filter and evaporate to dryness = TOTAL NEUTRAL FRACTION

discard urin

dissolve in 0.8 ml. glacial acetic acid add about 40 mg Girard's reagent T

leave the flask overnight at room temperature

or

heat the flask on boiling water-bath for 2 minutes

cool the flask in iced water

add 25 ml ice cold 0.55 N NaOH and transfer to a separating funnel

extract immediately with ice cold chloroform, 3 times with 25 ml., (chloroform = non-ketonic fraction)

discard chloroform

add 2 ml 40 vol per cent H_2SO_4 and 25 ml chloroform to the aqueous solution

2 hr later extract with the chloroform already present and twice more with 25 ml of chloroform

combine the chloroform extracts and wash by shaking 3 times with 10 ml. 0.1 N NaOH, and 3 times with 10 ml of water re-extract each washing with small amounts of chloroform

dry the extract with Na_2SO_4, filter and evaporate to dryness = KETONIC FRACTION

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ACKNOWLEDGEMENT

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INVESTIGATIONS ON THE URINARY EXCRETION OF CORTICOIDS AND 17-KETOSTEROIDS DURING THE ADMINISTRATION OF ADRENOCORTICOTROPHIC HORMONE (ACTH)*)

BY

MOGENS SPRECHLER

The adrenocorticotrophic hormone (ACTH) was isolated from an extract of the anterior lobe of the pituitary gland by *Collip et al.* in 1933 and 10 years later *Li et al* (1943) and *Sayers et al.* (1943), simultaneously, but independently prepared the pure protein hormone using as starting material, sheep and hog pituitary glands respectively Only quite recently, however, has it been possible to produce the hormone in quantities large enough for clinical experiments in normal subjects (*Mason et al*, 1948, *Forsham et al*, 1948, *Sayers et al.*, 1949) and in the treatment of various diseases, this followed the report by *Hench et al.* in 1949 of the astonishing effect of cortisone and ACTH in chronic rheumatic diseases. Already a fairly extensive literature on the subject has accumulated

*) Preliminary accounts of the investigations were given 1) at the meeting of the Danish Society for Endocrinology, Copenhagen, April 21, 1950, 2) at the Third Scandinavian Congress for Rheumatology, Oslo, May 25—27, 1950, 3) at the meeting of Scandinavian Society for Endocrinology First Congress, Stockholm, June 21—22, 1950, and 4) at the meeting of the Scandinavian Society for Clinical Chemistry and Clinical Physiology IV Congress, Finland, July 2—5, 1950.

and has been collected in the U. S. A. by *Mote* (1950). Here in Denmark, similar investigations are reported by *Brochner-Mortensen et al.* (1949), *Videbæk et al.* (1950), *Astrup et al.* (1950) and *Schwartz & Sonne* (1950).

ACTH seems to act as a catalyst in the adrenal cortex, stimulating the elaboration of the adrenal cortical hormones. This is followed by an increased secretion of these substances into the circulation. Cholesterol and ascorbic acid appear to be involved in this synthesis, as a single injection of ACTH into the test animal produces a depletion of these substances in the adrenals, the lowest values being reached after 3 and 1 hour respectively This can be used to determine the activity of ACTH preparations (*Sayers et al.*, 1943, 1949).

By the administration of ACTH to patients suffering from Addison's disease *Thorn et al.* (1948) have shown that the effect is due exclusively to a stimulation of the adrenal cortex, with a resulting increased elaboration of adrenal cortical steroids. By a determination of the quantity of these steroids, or more indirectly by a determination of their effect in the organism, it is possible to obtain some idea of the effects of ACTH in man. The best and most direct method would be a determination of the steroid content of the blood (*Hemphill & Reiss*, 1947, *Corcoran & Page*, 1948) but these analyses are very difficult and fairly large quantities of blood are needed. Consequently they are unsuitable for routine analyses. *Browne et al.* as early as 1943, demonstrated that the administration of purified ACTH to a male patient brought about an increased excretion of corticoids in the urine This has now been confirmed by numerous experiments, as has the increased excretion of another group of substances: namely the 17-ketosteroids In the female these steroids presumably originate almost entirely from the adrenal cortex

The first group of steroids consists essentially of the *glucocorticoids* which can be assayed biologically by their effect on glycogen deposition in the liver in adrenalectomized animals, chemically by their reducing properties or by their power to liberate formaldehyde by periodic oxidation (a number of bio-

logically inactive metabolites of the steroids mentioned are presumably included in the chemical determination) and finally, indirectly by their activity in the organism, where they bring about a decrease in the number of circulating eosinophil leucocytes, and as a rule, an increase of the uric acid/creatinine index in the urine.

At present no method is available for the direct measurement of the other well-known group of corticoids, the *mineralocorticoids*, but *Forsham et al.* (1948) believe that certain changes in the electrolyte balance indicate that these substances too are produced in increased quantities following ACTH stimulation, this appears to be confirmed by the investigations of *Conn & Louis* (1950), who found a decrease in the chloride and sodium concentration in the sweat during the administration of ACTH to patients.

The third group of steroids produced in the adrenal cortex are substances with sex hormone-like effects Of greatest practical significance are the androgens and their metabolites, the *17-ketosteroids.*

In the same way as in animal experiments, rapid depletion of cholesterol and ascorbic acid from the adrenal occurs, so the metabolic changes in man, which follow a single dose of ACTH given intravenously also take place very quickly (*Sayers et al.*, 1949). The maximum response is reached within a few hours and there is a return to the normal conditions in about 6—12 hours. The urinary excretion of steroids shows parallel variations. That the effect must be of very short duration is apparent from the fact that the amount of ACTH in the blood reaches a normal level very soon (less than 3 hours) after the injection, indicating a rapid inactivation of the hormone either by a binding to substances in the blood or the tissues, or by destruction. *Burns et al* (1948) demonstrated that following an intravenous injection of 240 mg of ACTH into a normal subject the excretion in the urine during the first 24 hours was less than 200 µg. Hence, to produce a continuous stimulation of the adrenal cortex, it is necessary to administer the ACTH in several divided injections during the course of

24 hours, usually every 6 or every 8 hours, after which time the normal state of the adrenal cortex is practically reestablished.

Under normal conditions it can be assumed that there exists an accurately balanced coordination between the anterior pituitary gland and the adrenal cortex and that there is a continuous production of ACTH and corticosteroids, which are necessary for the maintenance of life. There is no doubt, however, that the hormone of the adrenal medulla, i. e. adrenaline, can also affect this so-called pituitary-adrenal system, although there is good evidence, that this only occurs under conditions in which special demands are made on the body as summed up by Selye in his conception of »stress«. *Vogt* (1947) demonstrated an increased secretion of corticoids from the adrenals in a dog following injection of small amounts of adrenaline and it is now evident that this effect is due to a mobilization of ACTH from the anterior pituitary gland either by a direct effect on this gland or indirectly via the hypothalamus (*Hume & Wittenstein*, 1950). The nature of this regulating mechanism is by no means clear

As is apparent from the above, the effects of ACTH depend exclusively on the functional state of the adrenal cortex, while the effects of adrenaline are dependent on an intact (hypothalamic-) pituitary-adrenal system On the basis of these facts, *Thorn & Forsham* (1949) performed various tests for the evaluation of the pituitary and the adrenal cortical function. Amongst these may be mentioned 2 short-term tests based on the fact that 4 hours after an injection of adrenaline (0.3 mg.) or ACTH (25 mg) a reduction in the number of circulating eosinophil leucocytes occurs The first of these tests seems less suitable for determining the functional state of the adrenal cortex, since as has previously been mentioned, the evidence of an intact pituitary-adrenal system must be assumed and in addition adrenaline can probably mobilize only the pre-formed ACTH in the hypophysis, as suggested by Thorn & Forsham. The best method appears to be the so-called 48-hour ACTH test. 10 mg ACTH is administered intramuscularly

every 6 hours for 48 hours and during this period of time eosinophil counts are carried out and the excretion of 17-keto-steroids is determined in the two 24-hour urines.

The investigators have interpreted the results of such a test as follows:

»1) A low value for 17-ketosteroid excretion initially, which fails to rise appreciably under ACTH, accompanied by a poor fall in eosinophils, proves the absence of any adrenal cortical reserve and strongly suggests either a primary adrenal cortical insufficiency or a longstanding hypopituitarism.

2) A low value for 17-ketosteroids initially, which rises during ACTH therapy, accompanied by a progressive fall in eosinophils, is suggestive of moderate adrenal cortical atrophy secondary to pituitary failure.

3) A normal 17-ketosteroid level, which is increased to the upper limit of normal, with or without a fall in eosinophils indicates adequate adrenal cortical reserve A poor fall in eosinophils merely suggests the presence of an acute eosinophilia related to allergy.«

OWN INVESTIGATIONS

After the report from U. S. A (*Hench et al*, 1949) on the favourable effects of cortisone and ACTH on rheumatic diseases had reached Denmark, a number of similar experiments were carried out in various clinical departments here. Comprehensive metabolic studies have been performed in a number of these cases. In others, attention has chiefly been paid to the therapeutic effects In the Hormone Department of the State Serum Institute in Copenhagen we have had the opportunity of examining the urinary steroid excretion in the majority of the patients in this country in whom treatment with ACTH has been tried

The aim of the present study has been 1) to investigate whether, by a determination of the excretion of corticoids and 17-ketosteroids in the urine, a quantitative measure of the effects of ACTH in children and adults can be obtained. 2) to

examine any possible factors which may influence the response of the adrenal cortex to ACTH, and finally 3) if possible, to establish to what extent it is necessary, in a routine investigation, to carry out the above mentioned steroid analyses in the studies on the clinical effects of ACTH.

The clinical and other metabolic data obtained in these experiments are to be published elsewhere.

ACTH-preparations Several different preparations have been used during the study, all prepared from hog pituitary glands. 1) Cortrophin (Organon): 4 different batches, 2) Armour's ACTH, batch no. 32320. 3) ACTH obtained from two Danish factories. Each new batch from one of the factories (R. M. C.) has been standardized in the hormone department here by the method of *Sayers, Sayers & Woodbury* (1949) using the Armour La-1-A as a standard of reference (*Hamburger*, 1950). The stated potencies of the Armour- and some of the Cortrophin-preparations were also controlled.

Methods. The urine was collected in 24-hour periods without preservative and kept in the cold until the extraction which was, as a rule, carried out within the next 24—28 hours.

The corticoids were determined chemically by the reducing power of the ketonic fraction obtained by a Girard separation of the total neutral fraction extracted from urine acidified to pH 1 (*Sprechler*, 1950). The results are expressed in terms of the reducing power of desoxycorticosterone

The glucocorticoids were assayed biologically by the liver glycogen deposition test in mice, as described by *Venning, Kazmin & Bell* (1946), and the results are expressed in terms of μg of 17-hydroxy-11-dehydrocorticosterone

The 17-ketosteroids were measured by the micro-method of *Hamburger & Rasch* (1948). The results are expressed in terms of mg. androsterone, after a colour correction

In order to correct for the inaccuracies in the urine collection, determinations of creatinine excretion have been carried out in a number of cases. The excretion of this substances has proved to be subject to such large spontaneous daily variations, even when the patient was kept on a constant diet, that

it has been found unsuitable for the correction of inaccuracies which have practical significance.

Clinical material. The excretion of reducing corticoids as well as 17-ketosteroids in the urine has been investigated in 35 patients treated with ACTH. In 2 cases, cortisone was administered at the same time and the results will be described in a later publication; in 8 of the cases in which analyses have been made only every 4 days (*Brodthagen et al.*, 1950) no details are given here. In the following, the results obtained in the remaining 25 cases consisting of 2 children, 15 women and 8 men, will be described. In all these cases analyses have been carried out daily, as far as possible before, during and after the administration of ACTH

The most important data are given in Table 1. As can be seen, the material consists of practically all age groups, and includes various diseases, e. g., acute and chronic rheumatic diseases, leucemia, scleroderma, disseminated lupus erythematosus and Boeck's sarcoid The average excretion of corticoids and 17-ketosteroids is also given for those cases in which it has been possible to obtain such analyses before the commencement of treatment. In Fig. 1 the pre-treatment values for the 17-ketosteroids are plotted in the diagrams showing the excretion of the 17-ketosteroids in normal subjects as carried out by *Hamburger* (1948). The values for the corticoids cannot be shown in the same way, as the final limits have not yet been established. In the case of adult women, however, these values will undoubtly be found to be between 0.40 and 0 90 mg per 24 hours. As is seen in Fig 1, the excretion in a number of the cases is below the lowest normal limit or in the low normal range

The effect of the adrenal cortical function on the urinary excretion of steroids during the administration of ACTH

As we have had no opportunity of carrying out the 48-hour ACTH tests in cases with a normal adrenal cortical function as described by Thorn & Forsham, a short description will be given of the results of analyses carried out on the urine from

Table

Summary

'atient	Age	Sex	Diagnosis	Therapy in days	Total ACTH in mg	Average daily dose in mg	Corticoids mg 24 hrs (average) before ACTH	during ACTH	after ACTH
K.K.	35	F	Scleroderma	15	1820	121.3	0.72	3.16	0.81
D.L	28	F	»	23	2700	117.4	0.80	11.04	
M P.	43	M	Anchylos Spondylit	15	1420	94.7	0.81	4.39	0.79
B J	40	M	» »	14	1180	84.3	0.97	3.84	0.84
L R.	53	F	Chron Rheum Arthr.	15	950	63.3		2.64	0.70
K.G.L	50	F	» » »	5	190	38	0.45	2.70	0.69
J.J	47	F	» » »	11	400	36.4	0.44	0.83	0.85
T L	15	F	Acute Rheum Fever	11	392	35.6	0.61	3.42	0.50
A K	28	F	» » »	10	300	30	0.26	2.23	0.87
E.H.	48	F	Lup Erythem Diss	7	210	30	0.46	2.11	0.46
R F.	25	F	» » »	5	150	30	0.30	0.47	0.50
A T.	38	F	Chron Rheum Arthr.	4	120	30	0.44	0.68	0.45
C I.	57	M	Lymfatic Leucemia	8	230	28.8	1.07	4.20	1.43
V.I O	33	M	Boeck's Sarcoid	10	285	28.5	0.79	0.99	0.89
V O	16	M	Acute Rheum Fever	11	270	24.5	0.74	1.07	0.73
N N	45	M	Chron Rheum Arthr	9	210	23.3	0.73	1.62	0.70
A R	67	M	Myelogen Leucemia	8	130	16.3	0.64	1.15	0.86
E G	59	F	Chron Rheum Arthr	6	90	15	0.42	0.70	
E J.	54	F	Painful Shoulder	10	150	15	0.52	0.96	0.58
G.H	1²/₃	F	Collagen Disease	6	50	8.3	0.14	0.54	0.21
L.D	10	F	Still's Disease	76	572	Long term Experiments (small doses)	0.36		0.31
P P	59	M	Monocytic Leucemia	26 (4 Periods)	492.5				
F J.	40	F	Chron Rheum Arthr	125	1220				
H.H	59	F	» » »	58	885		0.72		
E R.	69	F	» » »	105	590		0.56		

of all cases

17-Ketosteroids mg/24 hrs (average)			First day's treatment					Second day's treatment					Maximum percentage increase	
				Corticoids		17-Ketosteroids			Corticoids		17-Ketosteroids			
before ACTH	during ACTH	after ACTH	ACTH dose mg/24 hrs	mg/24 hrs.	per cent increase	mg/24 hrs.	per cent increase	ACTH dose mg/24 hrs	mg/24 hrs	per cent increase	mg/24 hrs	per cent increase	Cort	17-ks
7 7	14.8	3 8	120	1.76	145	8 9	16	120	2 36	230	10 9	42	500	126
8 6	65 4	11.4	120	1.66	107	12 9	50	120	3 00	275	15 7	82	2075	947
9 6	21 0	9.7	100	2 15	105	17 6	83	100	2 88	256	16 8	75	925	230
9 7	31 0	10.3	100	2 05	112	13.4	38	100	3 00	210	25 1	159	452	315
2 7	11.7	3 9	25			3 1	15							
5 0	14 7	4 4	50	2.05	241	7 8	66	40	2 60	333	17.6	274	595	438
3 9	5 6	2 7	10	1 03	131	5.5	41	40	0 58	32	4 4	13	150	69
2.3	15 7	5 7	30	3.50	472	9 7	322	60	5.54	810	17 9	680	810	815
3 3	9 7	3 4	50	0 56	115	3 4	3	50	1.32	410	5 5	66	1350	375
3 6	17.0	4 2	30	0 37	−19	3.5	−3	30	0 54	17	4 1	14	650	1008
1.2	3 0	3 1	40	0 26	−4	1 7	42	40	0 36	39	1 4	16	178	340
3 0	4.3	2 6	40	0 73	66	4 2	40	40	0 69	57	4 1	36	66	47
3 0	6.5	2 3	10			2 5	−17	40	3 93	268	5 5	84	415	200
7 0	6 8	5 7	45	0 96	21	9 3	33	30	1.13	43	9 4	34	78	48
2.9	7 1	7.2	30	1 04	40	5 4	80	50	1.24	68	4 4	47	94	230
5 8	8 2	6 5	30	1 10	51	5 8	0	40	1 72	135	9 5	64	215	104
5 6	7 3	4.5	40	0 88	38	7 2	28	25	1 41	120	8 8	67	135	75
2 5	2 2		20	0 64	52	2 4	−4	20	0 74	76	3 0	20	76	20
3 0	6 7	5.5	40	1 44	176	8 2	173	20	1 31	152	10 0	234	223	234
0 40	0 42	0 35	6	0 26	88	0 38	−5	6	0 45	221	0 44	10	565	100
1 0		1 0	25	1 28	255	1 9	90	25	1.39	286	2.1	110	1525	450
6 4			30			10 5	64	50			23 0	260		260
5 5			25	4 65	545	7 9	44	25	4 38	510	14 2	158	730	295
2.8			30	0 76		3 9	39	40	1.49		9 1	225		496
3 7			*20*	*0 78*	*39*	*6 5*	*76*	*10*	*1 06*	*89*	*6 5*	*76*	*114*	*125*

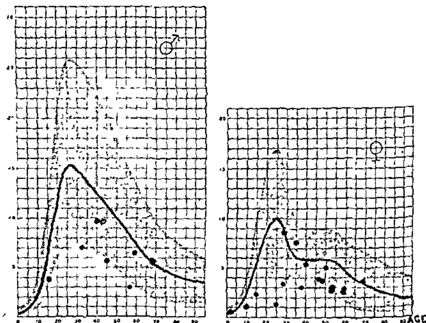


Fig 1

Average excretion of 17-ketosteroids during the pre-treatment period in the 25 patients. The black line shows the average output in normal subjects, and the stipled area gives the zone within which 97—98 per cent of normal values are falling, according to *Hamburger* (1948).

a patient under surgical stress, which produces similar changes in steroid excretion as are found during the administration of ACTH. The patient was a 32-year-old man with a gastric ulcer in whom a resection of the stomach had been performed. The urinary corticoids and 17-ketosteroids rose after the operation to a maximum (2.66 and 9.7 mg. per 24 hours respectively) which was reached on the second day (Fig. 2). This was immediately followed by a sharp fall to slightly below the pre-operative values, and finally there was a return to the original level. That the excretion did not begin to rise on the day of operation was no doubt due to an incomplete 24-hour urine collection, probably due to retention of urine. In this case the

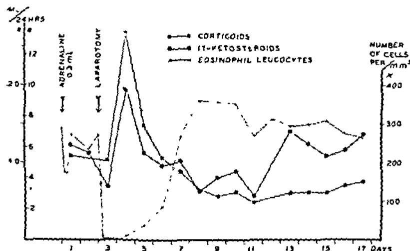


Fig 2
Changes in the excretion of corticoids, 17-ketosteroids and in circulating eosinophil leucocytes following major surgery

curve for the number of circulating eosinophils was seen to be a reflection of the curves obtained for the excretion of steroids.

Before the beginning of ACTH treatment there is no particular reason to carry out any of the tests for the evaluation of the adrenal cortical function It is very important, however, to establish the exact excretion level for the steroids and at least 3—4 days is necessary as a control period. These values give information on the functional condition of the adrenal cortex and, only in this way it is possible to evaluate the cortical response to ACTH subsequently.

Group 1 This group consists of 10 patients with a normal adrenal cortical function as measured by the response to ACTH The data for the treatment and the average values for the excretion of steroids will be found in Table 1 In all the cases daily analyses of the corticoids and 17-ketosteroids have been performed and the results are illustrated in Plate 1 Before the administration of ACTH the corticoid excretion was within the normal range with the exception of case 9 where in which

the excretion was only 0.26 mg./24 hrs. The excretion of 17-ketosteroids was also decreased in this case (3.3 mg./24 hrs) as well as in case 8 (2.3 mg./24 hrs.).

The dose of ACTH administered varied, but during the first two days' treatment between 30 and 100 mg. per 24 hours, divided into 3—4 injections, was given intramuscularly in all cases An increase in the urinary steroids was found on the first day and this increase continued progressively during the following days to reach a maximum after 3—5 days. If the same dose level was continued, a clear tendency towards plateau formation of the excretion curves was found in these short-term experiments. When the dose was reduced, however, the steroids fell to a lower level again. In most of the cases it was found that the steroid excretion reached the pre-treatment level on the first or second day after cessation of treatment. In a few cases, subnormal values were observed during the post-treatment period (case 8, 17 and 19). As is seen from Table 1 the percentage increase in the steroid excretion during the first and second day of the treatment was very varied, but as a rule it was most marked in the case of corticoids where the increase occurred as early as the first day. As previously stated, it should be pointed out that following the administration of ACTH, the increase in the steroid excretion takes place progressively, a response which is characteristic of cases with a normal adrenal cortical function.

Group 2. This group consists of 4 patients (7, 11, 12, 18) with a poor response to ACTH, indicating an impaired adrenal cortical function In no case, however, were there any symptoms of insufficiency Before going into further details about these cases it is of interest to find out what happens to the steroid excretion during ACTH administration in patients suffering from Addison's disease In Fig. 3 the results obtained in a 48-hour ACTH test carried out in such a case are shown. The patient was a 54-year-old woman, on whom a clinical diagnosis was made The excretion of corticoids and 17-ketosteroids in the control period was low: 0 24 mg. and 2 3 mg./

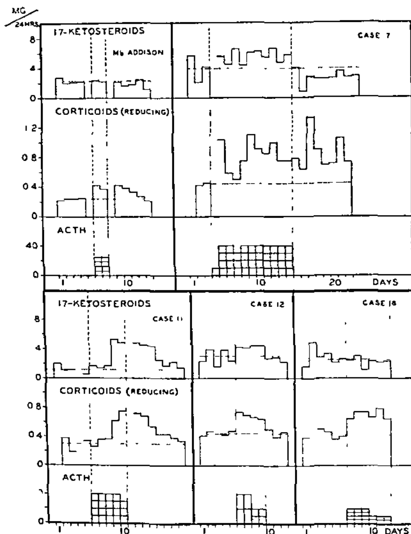


Fig 3

Effect of ACTH administration on the excretion of corticoids and 17-ketosteroids in a woman suffering from Addison's disease and in 4 cases with an impaired adrenal cortical function The horizontal dotted line represents the excretion level in the pre-treatment period

24 hrs. respectively. ACTH, 25 mg. was administered intramuscularly in 4 divided doses daily for two days. The corticoid excretion rose to 0.42 and 0.37 mg./24 hrs. (i. e. the lowest normal limit) and in the following 4 days, the excretion slowly decreased to the level found before the administration of ACTH. No significant changes were found in the excretion of 17-ketosteroids. Remnants of functional adrenal cortical tissue with corticoid-elaborating capacity seemed to persist and the insufficiency though not complete, was such that it produced clinical symptoms.

In Table 1 the data for the individual treatment of the cases referred to, are given, and in Fig. 3 the results of the steroid analyses are shown. In case 11 a very poor response was seen during ACTH administration especially with regard to the excretion of corticoids. The rise occurred very slowly beginning on the 3th and 4th day and the slow reaction continued subsequently so that the pre-treatment level was only reached after a week. This case is very similar to the case described above. In the other 3 cases a slight rise in the steroid excretion was also found in spite of adequate doses (30—40 mg./24 hrs.) of ACTH. In cases 11 and 12 the excretion of corticoids was within normal limits during the whole period of ACTH administration and in case 7 this only increased to the upper normal level. With the exception of case 12 it should *also be noted that the corticoid excretion only reached the* pre-treatment level slowly.

As mentioned above, there was in the first group one patient with a subnormal corticoid excretion, and two with a subnormal 17-ketosteroid excretion during the pre-treatment period; they all responded to the ACTH stimulation with a normal and strikingly increased excretion of steroids. On the other hand we have examined 2 cases, a 35-year-old woman (1) and a 28-year-old woman (2) both suffering from Scleroderma, in whom the excretion of corticoids as well as of that of the 17-ketosteroids was perfectly normal and on the same level during the period before the administration of ACTH. Both cases were treated with ACTH from the same

preparation, in doses of 120 mg. per day in 3 equal parts for the first few days. The results are shown in Fig. 4. In case 1 there was only a moderate increase in the steroid excretion, as the corticoids rose from 0.72 mg to a maximum of 4 33 mg./24 hrs, and the 17-ketosteroids from 7.7 mg. to a maximum of 17.4 mg./24 hrs. whilst the excretion in the other case reached very high values: the corticoids rose from 0 80 to 17.40 mg./24 hrs. and the 17-ketosteroids from 8.6 mg. to 90.6 mg./24 hrs. In addition it was found that the difference in the response of the adrenal cortex in the two patients was only clear on the third day of treatment, as the increase in the corticoid excretion was the same on the first day, and only slightly more marked in case 2 than in case 1 on the second day, namely: 3.00 and 2 36 mg /24 hrs. respectively, or 275 and 230 per cent. A possible explanation is that the adrenal cortex in case 16 had a relatively small potential reserve and, therefore, was unable to give an adequate response to the high dose administered during the prolonged period of treatment. The low values for the excretion of 17-ketosteroids in the post-treatment period must presumably have been due to an exhaustion of the adrenal cortices and it is probable that a continuation of the stimulation with high doses of ACTH would have been fatal

The relation between the dose of ACTH and the excretion of steroids.

In the available literature there is, as yet, no information as to amount of ACTH necessary to obtain an adrenal cortical stimulation sufficient to give a clinical effect. *Thorn & Forsham* (1949) used the changes in the number of circulating eosinophils as an index of the adrenal cortical response Following a single dose of 4 and 25 mg of ACTH the decrease in circulating eosinophils was found to be 50 per cent and 70—100 per cent respectively No particular attention has been given to amounts of steroids excreted.

The existence of a quantitative relationship between the dose and the amount of steroids excreted is of paramount sig-

24 hrs. respectively. ACTH, 25 mg. was administered intramuscularly in 4 divided doses daily for two days. The corticoid excretion rose to 0.42 and 0.37 mg./24 hrs. (i. e. the lowest normal limit) and in the following 4 days, the excretion slowly decreased to the level found before the administration of ACTH. No significant changes were found in the excretion of 17-ketosteroids. Remnants of functional adrenal cortical tissue with corticoid-elaborating capacity seemed to persist and the insufficiency though not complete, was such that it produced clinical symptoms.

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lations of the results found in the 2 cases which have been treated for some time with various doses of ACTH. In the first case (21) which is described in greater detail later (Fig. 10) an abnormally changed response of the adrenal cortex to ACTH developed after some time and the results in this period are omitted from the calculations. The average steroid excretion values for the various dose levels are shown in Table 2. When these values are plotted against the logarithm of the dose there is a linear relationship (Fig. 5). It is seen that the excretion of corticoids, as determined chemically as well as biologically, showed a definite increase at a dose level of about 5—6 mg./24 hrs. while in the case of 17-ketosteroids, this only occurred with 12—13 mg./24 hrs. In the other case (22, a male, age 59) ACTH was administered during 4 separate periods. The results of the steroid analyses, which in the case of corticoids only consists of the two last periods, are charted in Fig. 6 The average daily dose as well as the average 24-hour excretion of 17-ketosteroids has been calculated for each period (Table 2) and the log dose response curve (Fig 6) is also linear in this case, as the high value obtained during the second period of treatment must be attributed to the fact that this period of treatment follows very closely on the preceding one. It is clear from the curve that the excretion of 17-ketosteroids began to increase at a dose of 14—15 mg./24 hrs In our experience we find that the dose of ACTH necessary to increase the corticoid excretion in adults, is only slightly lower and on an average, about 12 mg./24 hrs. The average percentage increase in 17-ketosteroids during the period of ACTH administration was calculated in 18 cases and the results are plotted against the log of the average dose of ACTH (Fig 7). There is a close agreement with the above-mentioned results. Although we have not had much experience in this laboratory of the changes in the number of circulating eosinophils during ACTH treatment, it is our impression that this occurs even with considerably lower dose levels. In none of the cases was a clinical effect observed without an increase in steroid excretion.

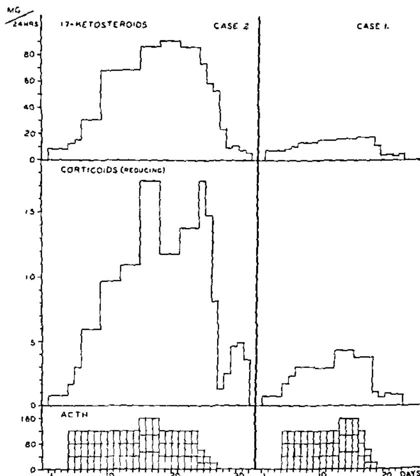


Fig 4

Effect of high dosage of ACTH to 2 women with scleroderma Note the normal steroid response during the first 2 days' treatment followed by marked differences in spite of a dosage of the same range

nificance in determining whether steroid excretion can be used as an index of the action of ACTH.

We have not yet had an opportunity of carrying out systematic experiments to solve this important question. Some evidence on the relationship can be obtained by making calcu-

lations of the results found in the 2 cases which have been treated for some time with various doses of ACTH. In the first case (21) which is described in greater detail later (Fig. 10) an abnormally changed response of the adrenal cortex to ACTH developed after some time and the results in this period are omitted from the calculations. The average steroid excretion values for the various dose levels are shown in Table 2. When these values are plotted against the logarithm of the dose there is a linear relationship (Fig. 5). It is seen that the excretion of corticoids, as determined chemically as well as *biologically, showed a definite increase at a dose level of about* 5—6 mg./24 hrs. while in the case of 17-ketosteroids, this only occurred with 12—13 mg./24 hrs. In the other case (22, a male, age 59) ACTH was administered during 4 separate periods. The results of the steroid analyses, which in the case of corticoids only consists of the two last periods, are charted in Fig 6. The average daily dose as well as the average 24-hour excretion of 17-ketosteroids has been calculated for each period (Table 2) and the log dose response curve (Fig. 6) is also linear in this case, as the high value obtained during the second period of treatment must be attributed to the fact that this period of treatment follows very closely on the preceding one It is clear from the curve that the excretion of 17-ketosteroids began to increase at a dose of 14—15 mg./24 hrs In our experience we find that the dose of ACTH necessary to increase the corticoid excretion in adults, is only slightly lower and on an average, about 12 mg./24 hrs The average percentage increase in 17-ketosteroids during the period of ACTH administration was calculated in 18 cases and the results are plotted against the log of the average dose of ACTH (Fig 7) There is a close agreement with the above-mentioned results. Although we have not had much experience in this laboratory of the changes in the number of circulating eosinophils during ACTH treatment, it is our impression that this occurs even with considerably lower dose levels. In none of the cases was a clinical effect observed without an increase in steroid excretion.

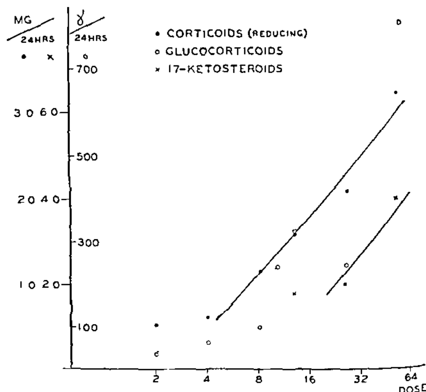


Fig 5

The average excretion values for reducing corticoids (●), glucocorticoids (○) and 17 ketosteroids (x) plotted against the dose in mg. ACTH (log scale) in case 21

As mentioned in the introduction there is reason to assume that the greatest and most constant effect of ACTH is obtained when the hormone is administered in divided doses during the 24-hour period instead of giving the whole daily dose in one injection *Thorn & Forsham* have stated that a single dose of 25 mg. ACTH administered intramuscularly produces a maximum stimulation of the adrenal cortex as measured by the decrease in the number of circulating eosinophils, while *Li* (1949) has stated that the adrenal cortex only utilizes at the most 10 mg per injection and probably even less. Here, too, it must be remembered that we have not yet carried out

Table 2

Case	ACTH mg/24 hrs	Number of days	Average 24-hour excretion		
			17-Ketosteroids mg	Corticoids (reducing) mg	Glucocorticoids µg
21	0	6 (pre-treatment)	1.0	0.36	43
	4	6	1.2	0.61	67
	8	5	(2.2)	1.16	198
	12.5	4	1.8	1.58	324
	25	4	2.1	2.09	342
	50	4	4.2	3.22	825
22	Average	Period			
	0	Control	6.4		
	27	I	16.1		
	12.5	II	(10.0)		
	19.0	III	10.14		
	16.0	IV	7.2		

Effect of various doses of ACTH on the urinary excretion of steroids in 2 patients. The average excretion value is calculated for each dose level in case 21 and for each period of treatment in case 22.

any systematic experiments. In order to elucidate this problem, a comparison of the adrenal cortical response obtained in two groups of patients will be given. The first group consists of 3 cases 2 (Fig. 4), 3, 4 and 5 (Fig. 8). Case 5 was treated with 75—100 mg. of ACTH administered in one daily injection, case 2 and case 3 received 120 and 100 mg ACTH/24 hrs. respectively divided up into three doses of 40 mg. in case 2 and 20, 40 and 40 mg. in case 3. The other group consists of cases 6, 8, 9, 10, 16 and 19 illustrated in Plate 1. In these cases the dose was around 30—40 mg./24 hrs It is evident that the utilization of the hormone administered was considerably greater in the last-mentioned group than in the first group. On the other hand, the utilization must be a great deal more than 10 mg. given in a single injection. This is most clearly seen in case 5 who received a high daily dose in one single injection. The corticoids increased to a maximum of 4.25 mg. and the 17-ketosteroids reached 17.9 mg./24 hrs. (the upper normal range

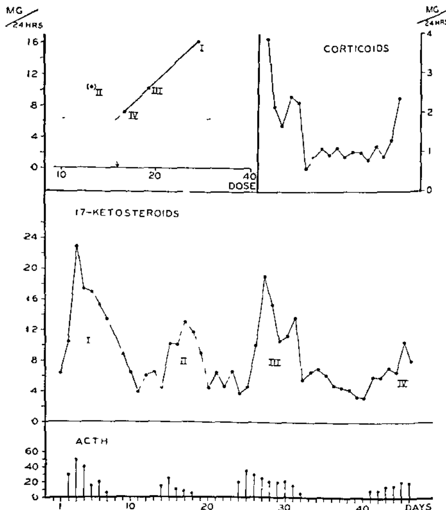


Fig 6

Effect of ACTH administration during 4 periods on excretion of 17-ketosteroids and corticoids The average excretion value for each of the periods is plotted against the average dose of mg ACTH, log scale (Case 22)

for a woman of her age is 0.90 and 9.0 mg. respectively). Such a high increase in the urinary steroids was only seen in our cases when the 24-hour dose was at least 30 mg The utilization of the single doses of ACTH in case 2 corresponded close-

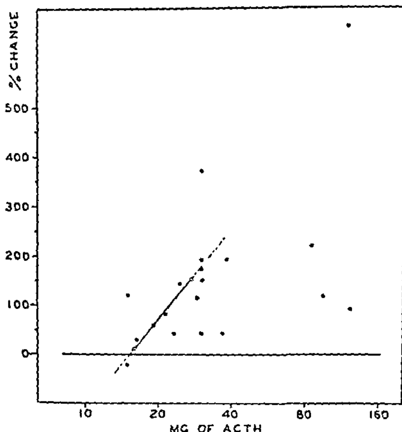


Fig 7

The average percentage increase in 17-ketosteroids during the period of ACTH administration to 18 patients. The horizontal line represents the pre-treatment level. The »dose-response curve« shown in the Figure is obtained by calculations of the results illustrated in Fig. 6. Ordinate: The average percentage change during the ACTH period. Abscissa The average dose of mg ACTH (log scale)

ly to the above mentioned value though somewhat lower in case 3. From the present material it is not possible to establish the exact size of the largest single dose which can be completely utilized but it seems to be of the order of 20 to 30 mg., presumably with rather large individual variations.

In the above mentioned 3 cases subjected to a high dosage

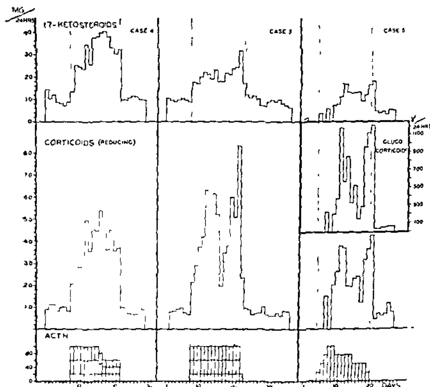


Fig 8

Effect of high dosage of ACTH on the urinary excretion of corticoids and 17 ketosteroids in 3 cases. A decreased corticoid response is seen in case 3 and 5 after 8—9 days' treatment

of ACTH a peculiarity in the course of the steroid excretion curves was observed. In all the cases a marked rise in the excretion of steroids was seen during the first few days of treatment, indicating an adequate adrenal cortical reserve, but after 9—10 days a striking decrease in the excretion of corticoids occurred. In case 3 a decreased eosinophil response occurred at the same time. In case 5 a parallel decrease was seen in the excretion of the biologically assayed glucocorticoids and the 17-ketosteroids. In this latter case the ACTH dose was reduced at this time and a new steep increase in the excretion was observed. It was believed that an overdosage had resulted in ex-

haustion of the adrenal cortex. If, however, only a limited amount of the daily dose of ACTH referred to was utilized, such an explanation is not particularly satisfactory, nor does it explain the fact that a marked increase in the urinary steroids also occurred in case 3 in spite of the continued and unchanged high dosage of ACTH.

The effect of ACTH on the ratio between corticoids and 17-ketosteroids excreted in the urine.

In several cases a definite parallellism is found between the excretion curves for corticoids and 17-ketosteroids during the administration of ACTH especially with regard to the variations observed from day to day It is remarkable, however, that in most cases the percentage increase above the pretreatment level is more marked in the excretion of corticoids than of 17-ketosteroids. This difference is often found on the first day of treatment, and in several cases the corticoids reached the maximum point of the excretion curve before the 17-ketosteroids. These differences in the response are clearly illustrated in case 6, 8 and 13 (Plate 1) and by the data given in Table 1, In case 13 the 17-ketosteroids increased from 3.0 mg. to the maximum 9 0 mg /24 hrs. which is within the normal range, while the corticoids increased from 1.07 to 5 50 mg /24 hrs. Oedema, however, developed at this stage and treatment had to be discontinued as the complication was considered to be due to an overdose of ACTH.

Only in one case (10) was the opposite observed, as the 17-ketosteroids increased to a maximum of 39 9 mg /24 hrs. or 1008 per cent while the corticoids reached 3 44 mg /24 hrs. or 650 per cent.

These differences in the excretion of the two groups of steroids suggest that they are produced independently of each other in the adrenal cortex *Venning & Kazmin* (1946) show that the excretion of corticoids reach the adult level during the first few years of life, while the 17-ketosteroids rise slowly with increasing age We have therefore been particularly interested in determining whether these characteristic dif-

ferences can also be seen during ACTH administration. Because of inadequate quantities of ACTH at our disposal it has unfortunately only been possible to carry out two such experiments.

In the first experiment (case 20, a girl, age 20 months) ACTH was administered during a period of 7 days with a total daily dose of 6 mg. during the first 6 days, and of 12 mg. on the 7th day, all these doses being divided into 4 injections. During the treatment the reducing corticoids increased from 0.14 to 0.94 mg./24 hrs. and the glucocorticoids increased from 21 μg to 185 μg/24 hrs. The 17-ketosteroids were at first decreased, and a slight rise was observed only during the last two days of treatment (Fig. 9).

In the second experiment (case 21, a girl, age 10 years) ACTH from the same preparation was given in various doses. Calculated on a weight basis, however, the dose was of a similar range as used in the first experiment. During the first period of treatment (Fig 9) a marked increase in the 24-hour urinary excretion of corticoids was observed: The reducing corticoids rose from 0.36 to a maximum of 5.49 mg. and the glucocorticoids from 43 μg to 585 μg whilst during the same period the 17-ketosteroids only increased from 1.0 to 2 5 mg per 24 hours After treatment for 14 days and when the dose of ACTH had been increased to 50 mg. the 17-ketosteroids rose to 4 7 mg /24 hrs. By comparing the steroid excretion values found in case 20 with those usually observed in cases of adult subjects (for example case 5) the values for the 10 years old girl appear to lie midway

The treatment of this patient was continued for a period of 76 days During 3 periods a relatively high dosage of ACTH was given, while only 2 mg. per day was administered for one month between the first two periods and for some time after the second period. Numerous steroid analyses were performed and the results obtained are shown in Fig. 10 During the period of low dosage the excretion of corticoids was slightly but constantly raised while the excretion of 17-ketosteroids during the first period varied a little from day to day though

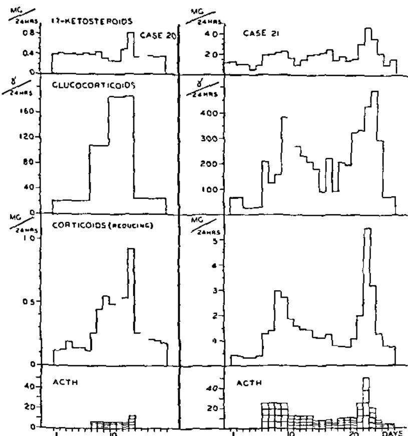


Fig 9
Effect of ACTH administration on the excretion of reducing corticoids, glucocorticoids and 17-ketosteroids in 2 girls (age 1²/₃ and 10 years).

the average level was unaltered After 50 days' treatment, however, it is interesting to note that there was a distinctly increased excretion of 17-ketosteroids, in spite of the continued low dosage of ACTH This increase was still more marked during the 14 days of low dosage which followed the second period of high dosage The adrenal cortical response, as measured by the corticoid excretion, was rather poor during the second period of high dosage (a decreased eosinophil response



Fig 10

Effect of ACTH administration on the excretion of corticoids and 17-ketosteroids in a long-term experiment with a 10-year old girl The results are illustrated as percentage change from the pre-treatment level

was also found and the condition improved only slightly, (*Astrup et al*, 1950)). Subsequently, the treatment was discontinued for 20 days during which pre-treatment values for the steroids were again found. When after this time the third period of high dosage was initiated the adrenal cortical response was similar to that found at the beginning of the treatment

The excretion of steroids during prolonged administration of ACTH

An intensive study of the clinical effect of ACTH in various diseases has been performed for one year all over the world and it is now evident that the beneficial effects generally cease shortly after discontinuation of the ACTH administration, at any rate in the majority of cases which come under the heading of chronic rheumatic diseases. Thus if there is to be any hope of a lasting improvement, the treatment should undoubtedly be continued over a long period.

The prerequisites for beneficial effects with ACTH in such long-term experiments are first of all that the adrenal cortical response should be maintained and secondly that antihormone formation should not take place

In a few cases we have had the opportunity of studying the steroid excretion in long-term experiments and the results in 4 such cases are described Attempts have also been made in 3 cases to investigate the effects of small doses

Case 21 has already been mentioned The treatment apart from an interruption of 20 days was given over a period of 96 days.

Case 23 (female, age 40) received treatment for 130 days. ACTH was administered in doses of 25 mg daily for 3 days, 12 5 mg daily for 2 days and subsequently 5 mg. daily for a long period The results are shown in Fig 11. A striking increase in the excretion of corticoids from 0 72 mg to 4 65 mg./24 hrs immediately occurred whilst the 17-ketosteroids increased from 5 5 mg. to a maximum of 21 4 mg /24 hrs, which is twice as much as the upper normal limit During the following period of low dosage the excretion level of the corticoids was still slightly raised but tended to fluctuate with a rythm of 14 days duration. The excretion of 17-ketosteroids varied a great deal and the average level was not definitely raised As the clinical effect during this period was remarkably good the treatment was stopped on the 72nd day Immediately the steroids fell to values a little below the pre-treatment level After a week, low doses of ACTH were again given but without any effect on the urinary steroids When the amount of ACTH was increased to 50 mg per day for 4 days, only a very slight increase in the corticoid excretion was found and an even slighter increase in the 17-ketosteroids After a week the response had almost disappeared

Case 24 (female, age 59) received periodic treatment (Fig 11). The initial rise in the steroids was not so marked as in case 23. In this patient a poor response to the treatment was seen after 40 days, but after this there was no increase at all, even after 100 mg. of ACTH for one day



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there was a fall in the steroid excretion to low values (Fig. 11) In the meantime a change had been made to a new batch of ACTH and when this was standardized it was found to be only half as active as the previous batch thus explaining the findings in the steroid excretion in this case.

DISCUSSION

As the effects and many of the metabolic changes observed during the administration of ACTH will be, or have been reported elsewhere the discussion will be restricted to the results of the steroid analyses.

In investigations on the therapeutic effect of ACTH it is in most cases possible to obtain an evaluation of the functional condition of the adrenal cortex by a determination of the urinary steroids before and during the first two days of treatment.

Tests for the evaluation of the functional condition of the adrenal cortex can be very useful in clinical diagnosis, particularly in cases with hypofunction of the gland. This condition may partly be primary and due to disease in the adrenal gland itself, and partly secondary, due to diseases in the hypophysis and hypothalamus, or to other disturbances in the pituitary-adrenal relationship. *Thorn et al.* (1949) who have worked out the adrenaline and ACTH tests already referred to, seem to attach most importance to the changes in the number of circulating eosinophil leucocytes, while *McIntosh et al* (1950) emphasize that steroid analyses offer more reliable information as a result of studies on the effects produced in an 8-hour ACTH test in 12 cases with pituitary tumours, all of which showed evidence of hypofunction of the anterior pituitary gland. As we have not as yet sufficient material to deal with both of these tests for adrenal cortical response, it is impossible to make any contribution to this question. On the other hand there seems to be good reason for modifying the 48-hour ACTH test to include a determination of the urinary corticoids, as further information has been obtained in some cases, in the

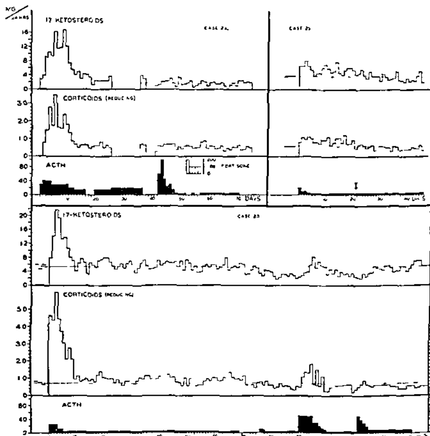


Fig 11
Development of a refractory state in long-term experiments with ACTH

Case 22 (male, age 59) received treatment for 26 days in 4 periods with free intervals of 5—7 days' duration. A good response was obtained and it continued unchanged throughout the periods (Fig. 6).

Case 25 (female, age 69) was also treated for a long time with small doses of ACTH This case is mentioned here in order to emphasize the importance of using only standardized preparations in all these experiments. ACTH was administered in doses of 20, 20 and 10 mg. during the first 3 days and subsequently in small doses At one point (↓), as in case 23,

In several cases it has been possible to obtain a reasonably good and reliable measure of the functional state of the adrenal cortex by a determination of the urinary steroids during a period of 3—4 days before the treatment (Fig. 1). In a few cases, however, in which the steroids were excreted at a low level during this control period, the response to ACTH was found to be entirely normal. Hypopituitarism of short duration in which an atrophy of the adrenal cortical cells has not yet taken place appears to be the most reasonable explanation in such cases. As typical examples case 8 and 9 should be mentioned. Both of these patients were suffering from severe stress due to the effects of acute disease (*Videbæk et al.*, 1950) and an increased elaboration and excretion of cortical steroids was therefore to be expected. As the immediate response to ACTH was normal, exhaustion could be excluded. The most reasonable explanation therefore, must be an acute failure occurring at a higher level in the system, most probably in the link: hypothalamus-hypophysis. One might be led to believe that acute rheumatic fever either actually develops from, or is in some way closely related to such a failure in this system through which the adaptation to stress is established. This would also explain why ACTH treatment has such a favorable effect in this disease.

When studying the clinical effects of ACTH treatment or the adrenal cortical response in tests for the evaluation of the functional condition of the gland, it is of course of paramount importance that an adequate dose of ACTH be given. Up till now, however, only a few attempts have been made to establish the dosage suitable for treatment with this hormone. According to most of the reports from U. S. A. fairly large doses have been used there, mostly 75—100 mg. per day or even more in some instances. In other centres limited amounts of hormone have been available and for this reason it has been necessary to use the smallest dose which is effective clinically. The problem as a whole is still unsolved and is further complicated by the fact that as yet little is known about the amount of ACTH necessary for the elaboration of

same way as the corticoids seem to give a more reliable indication of the adrenal cortical response to ACTH than do the 17-ketosteroids. According to our experience the results of steroid analyses performed in a 48-hour ACTH test or during the first two days of ACTH treatment can be explained as follows: 1) If no increase of steroids is found, there is complete insufficiency. 2) A slight increase in the urinary steroids on the first day which fails to rise any further on the second day or is possibly only found on the second day, suggests a relative insufficiency (hypofunction) and also a poor adrenal cortical reserve. 3) Marked and progressive increase beginning from the first day, and frequently amounting in the case of the corticoids to several hundred per cent, and ranging to the upper normal limit or a little above for the 17-ketosteroids indicates a normal adrenal cortical function and an adequate reserve. Furthermore the findings illustrated in Fig. 3 (Addison's disease) indicate that there is a dissociation of the adrenal cortical function with a complete androgenic insufficiency and a relative insufficiency with regard to the corticoids.

The 4 cases with hypofunction showed no symptoms of insufficiency and should be classified under the second group in which most of the cases are presumably secondary to a hypopituitarism or to a failure in the link. hypothalamus-anterior pituitary gland. In the cases mentioned it is interesting to note that on cessation of treatment the steroids only returned to the pre-treatment level very slowly from the slightly raised values obtained during stimulation This suggests a regeneration of the cortical cells resulting in an improved function which cannot, however continue for more than a few days after stimulation has ceased In such cases treatment with ACTH should, therefore also be given provided the proper treatment is preceeded by a period during which the adrenal cortical function is carefully built up by small but adequate doses of ACTH In all probability this will also prove to be of great practical importance as a means of preventing shock and other complications following major surgery in cases of an impaired adrenal cortical function (*Roche et al*, 1950).

ments on a baby. It is, however, our experience that this dose is somewhat higher in the case of adults and probably only slightly lower than the dose mentioned above for the 17-ketosteroids. It is reasonable to suppose that the production and secretion of endogeneous ACTH is suppressed when ACTH is administered and that if this suppression is complete the minimum effective dose should be equal the amount produced by the hypophysis. If the suppression is not complete the remaining amount of endogenous ACTH should be added to the minimum effective dose in order to determine the capacity of the hypophysis. c) The 24-hour excretion in normal women ranges from 0.40 to 0.90 for corticoids and 2—10 mg. for 17-ketosteroids, in the case of the latter there are marked variations which are dependent on age (*Hamburger*, 1948). These figures are comparable with the figures given for the extra excretion found, when a dose of about (20)-30 mg per 24 hours of ACTH was given to adults with an intact adrenal cortical function.

From the above it can be concluded that administration of ACTH in doses ranging to an upper limit of about 15 mg per 24 hours is within the normal physiological limits. At the same time it should be pointed out that the minimum therapeutic effective dose in several cases appears to be larger than that referred to above but in some cases it appears possible to obtain a therapeutic effect with such a small dose.

All the above calculations are based on the fact that the amounts of steroids excreted were found to be a function of the dose employed. In the case of the 17-ketosteroids this is confirmed by *Luft* (1950) who found that the relationship was entirely similar to that described here. *Hills & Thorn* (1948) found a similar relationship between the fall in the number of circulating eosinophils and the dose of ACTH given in a single injection. This test seems to be very sensitive in so far that the response to very low doses (about 4 mg) is seen even in adults, and experience has shown that the eosinophils reach zero or very low values after the administration of 25 mg. of ACTH for one or a few days. In many of the cases

the cortical steroids required under different conditions. It is therefore of interest to sum up the known facts about this problem and compare them with the results of our own experiments. *Sayers et al.* (1949) suggest that the pituitary gland in a normal adult person contains about 20 mg. ACTH and that 50 per cent can be liberated under conditions of severe stress, provided the conditions are similar to those found in experiments in animals. *Corcoran & Page* (1948) have stated that only 0.05 per cent of the corticoids in the blood which pass through the kidneys is excreted in the urine, and this amount was found by *Venning & Kazmin* (1946) to average about 50 µg per 24 hours in the case of normal adult subjects. Provided that a continuous elaboration and secretion of these steroids go on in the adrenal cortex it can be roughly estimated that about 100 mg. are produced in 24 hours allowing of course for certain variations which are dependent on the demands made on the organism. Assuming that this elaboration entirely depends on the amount of ACTH secreted should be of the order of 16 mg. as *Thorn & Forsham* (1949) showed that this amount of ACTH is equivalent to 100 mg. compound E as measured by their effect on the circulating eosinophils.

A number of these calculations must be considered as only approximate but nevertheless the results of our experiments show them to be highly probable as can be seen from the following a) By studying the alterations in the urinary steroids in the case subjected to a major operation (Fig. 2) and comparing the results with the dose-response curve in case 22 (Fig. 6) it will be seen that the increase in 17-ketosteroids on the second day after the operation corresponds to the increase produced by a dose of about 20 mg. ACTH administered during 24 hours. b) By comparing the results obtained in cases 21 (Fig. 5) and 22 (Fig 6) it will be seen that the minimum effective dose necessary to produce an increased excretion of 17-ketosteroids in both cases was of the order of 12—14 mg. per 24 hours. With regard to the corticoids the minimum effective dose in case 21 was found to be only 5—6 mg. which is similar to the amount given by *Venning* (1950) in experi-

at the same time increased demands on the cortical hormones, as occurs in cases of infections, trauma and other severe forms of stress. *Sayers & Sayers* (1948) have dealt with this subject in great detail. It has been mentioned that the peculiar two-peak excretion curves in cases 2, 3 and 5 can perhaps be explained by a relative overdosage. As can be seen the single dose in each of the three cases was very high, and therefore it seems possible that the whole of the potential reserve of the adrenal cortex was used up after each injection. In all the cases the decreased response occurred on the 9th—10th day of treatment, and the possibility of allergic phenomena cannot be excluded.

The dissociation between the excretion of corticoids and 17-ketosteroids during ACTH administration is clearly indicated by the greater percentage increase in corticoids, which are also often the first to reach the maximum level. This dissociation appears to be very characteristic and has been previously observed by *Sayers et al.* (1949) who discussed the possibility of a conversion of corticoids into 17-ketosteroids by a degradation in the liver or other tissues. The increase in 17-ketosteroids during ACTH treatment and the different excretion rate of the steroids mentioned above could be explained in the following manner. *Thorn & Forsham* (1949) observed a small increase in the urinary 17-ketosteroids during the administration of cortisone which we have also observed in our laboratory. Only very small quantities, however, seem to be converted even when large amounts of cortisone are administered, and it is therefore difficult to believe that the greatly increased excretion of 17-ketosteroids often seen during ACTH treatment could arise from an increase in the corticoids

In the discussion of this problem the findings made in Cushing's syndrome are worth recalling. In several of these cases a greatly increased excretion of corticoids is found while the 17-ketosteroids in many of the cases do not exceed the highest normal limit. Exactly similar conditions were found, for example, in cases 13 and 16 in which the corticoid excretion amounted to 2.90 and 5.50 mg. per 24 hours respectively

treated with ACTH the practical value of this test is greatly limited because of the narrow range of dosage.

The total daily dose of ACTH has usually been administered in divided doses over 24 hours without any systematic investigation as to whether this method of administration is necessary. In experiments on normal human subjects *Recant et al.* (1950) found a relative refractory period up to 8 hours after a single injection of 25 mg. ACTH intramuscularly, which corresponds closely to the changes found in the adrenal content of ascorbic acid and cholesterol following ACTH stimulation. A consideration of the results of our own investigations on the basis of these fundamental facts, suggest the following conclusions: in the case of *normal adrenal cortical reserve* and with a daily dose which does not exceed the maximal single dose of utilization (i. e. about 20 mg.) per 24-hour, the response as measured by the urinary steroids appears to be the same whether the hormone is given in a single injection or in divided doses over the 24 hours. If larger amounts of ACTH are administered the greatest effect is obtained by dividing the 24-hour dose into injections given every 6—8 hours, as can be seen by comparing for example, cases 2, 5 and 10. In the case of *reduced adrenal cortical reserve* there is no doubt that considerably smaller quantities of ACTH are utilized with each injection, but it is, as yet, impossible to decide whether the relative refractory period is of the same duration as that found under normal conditions, or whether it is longer. As previously mentioned it is possible in such cases to promote the activity of the adrenal cortex by prolonged stimulation with small doses of ACTH, thus increasing the amounts of hormone which can be utilized.

In the clinical use of ACTH, the question arises as to whether there is a possibility of overdosage i. e. whether this might cause a fatal exhaustion of the adrenal cortex Under normal conditions and in the case of a normal functional state of the adrenal cortex there is probably no great danger of this occuring. In cases of an impaired function there is undoubtedly a risk especially in those cases in which the organism makes

as compared with those in adults, showed that the 17-ketosteroids only increase very slightly and that the younger the child the smaller the increase, while the rise in corticoids corresponds to that found in adults. These results definitely indicate that it must be the capacity of the adrenal gland itself to elaborate 17-ketosteroids which is only little developed in children. The total result of the investigations in case 21 (Fig. 10) supports this conception, as the 17-ketosteroids were decidedly increased after 50 days of ACTH treatment, in spite of the fact that the dose was unaltered, thus suggesting an incipient maturity of the androgenic or 17-ketosteroid producing function of the adrenal cortex. Further investigations are necessary for the final clarification of these questions In such experiments it is undoubtedly important to take into consideration the actual size of the dose and it would be reasonable to calculate this on basis of the body weight in order to be able to compare the results obtained in children and in adults.

In the available literature only a few determinations of the steroid excretion in long-term experiments with ACTH are reported *Adams* (1950) observed a reduced eosinophil response in several cases after 20—30 days' treatment and later no response at all. This is described as »escape from ACTH« though it has not been possible to give a definite explanation for this phenomenon. *Mason* (1950) studied the steroid excretion in one case treated with ACTH for a period of 87 days and did not find any change in the response. In the present material the »escape phenomenon« was observed in two cases (23 and 24); in case 23 after the administration of ACTH for 70 days, and in case 24 after about 40 days. In the first case the sedimentation rate began to increase at the same time. In both these cases there were no symptoms of insufficiency of the adrenal cortex, and exhaustion seems to be excluded. The most likely explanation of the phenomenon would seem to be the formation of antibodies (antihormones?). *Forsham* (1950) has observed decreasing eosinophil responses to ACTH administered in repeated periods. In case 22 the treatment was given during 4 periods but no change was found in the response as

while the 17-ketosteroids were not increased beyond the normal range. The reversed ratio between the groups of steroids which is seen in cases of the so-called adrenogenital syndrome has not been observed during ACTH treatment, but in one of the cases (case 10) there was a greater percentage increase in the excretion of 17-ketosteroids than in that of corticoids. As the ACTH preparation used in this case produced a rather marked effect on the 17-ketosteroid excretion in some other cases, we have considered the possibility of a qualitative difference in the different ACTH preparations i.e. whether ACTH is a homogeneous material or whether it consists of several trophic factors, each of which is concerned with the elaboration of corticoids and 17-ketosteroids respectively. This problem has been previously dealt with in detail by *Reiss* (1948) and *Sayers & Sayers* (1948) and the latter investigators in particular stress the unitarian conception as the most likely Investigations on steroid excretion in children seem to be particularly suitable for the further elucidation of the question since there is a distinct difference in the excretion ratio between the corticoids and the 17-ketosteroids, as compared with the values found in adults. This does not appear to be due to differences in the intermediate steroid metabolism, as stressed by *Talbot et al.* (1950), who point out that testosterone propionate given to children is converted into and excreted as 17-ketosteroids in the same way as in the adult Therefore as stated by the authors mentioned above only two other explanations seem to be possible. 1) that ACTH consists of two different trophic factors, one stimulating the elaboration of corticoids and the other concerned with 17-ketosteroid production, the latter factor being produced in larger amounts only with increasing age, and 2) that the capacity of the adrenal cortex to form 17-ketosteroids or their precursors is only developed slowly, reaching a maximum at about the age of puberty. *Talbot et al* found that administration of ACTH to children had the same effect on the excretion of 17-ketosteroids as in adults, and this strongly supports possibility 1), whilst the results of our experiments in children (case 20 and 21)

as compared with those in adults, showed that the 17-ketosteroids only increase very slightly and that the younger the child the smaller the increase, while the rise in corticoids corresponds to that found in adults. These results definitely indicate that it must be the capacity of the adrenal gland itself to elaborate 17-ketosteroids which is only little developed in children. The total result of the investigations in case 21 (Fig. 10) supports this conception, as the 17-ketosteroids were decidedly increased after 50 days of ACTH treatment, in spite of the fact that the dose was unaltered, thus suggesting an incipient maturity of the androgenic or 17-ketosteroid producing function of the adrenal cortex. Further investigations are necessary for the final clarification of these questions. In such experiments it is undoubtedly important to take into consideration the actual size of the dose and it would be reasonable to calculate this on basis of the body weight in order to be able to compare the results obtained in children and in adults.

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measured by the urinary steroids. It should be stressed that the eosinophil response is a secondary effect of ACTH and that if it fails to appear this does not necessary mean a decreased response of the adrenal cortex to ACTH but may be due to an altered reactivity of the »end organs« to the corticoids

In those cases referred to in which only a few steroid analyses have been made during treatment with ACTH no particular information has been obtained except that it can be ascertained whether the response of the adrenal cortex is constant or changes gradually. This is of special interest in long-term experiments. In the case of high doses of ACTH in short-term experiments it should be of interest to investigate the steroid excretion throughout the whole period of treatment. In such experiments the method of study used in case 1 and 2 could be used in the future, and an overwhelming large number of analyses thus avoided. In these cases the urine was collected over periods of 3 days before, and during the treatment. Daily analyses were performed on the first and second day and from the data thus obtained the functional capacity of adrenal cortex could readily be ascertained. The potential functional reserve, as measured by the maximum increase during the following period of treatment, cannot, as is seen in the two cases, always be assessed by the analyses carried out in the two first days.

SUMMARY

The excretion of corticoids and of 17-ketosteroids in the urine is determined before, during, and after, the administration of ACTH to 25 patients including 2 children, 15 women and 8 men suffering from various diseases (chronic rheumatoid arthritis, acute rheumatic fever, leucemia, scleroderma, disseminated lupus erythematosus and Boeck's sarcoid).

1) a. The normal course of the steroid excretion curves during ACTH administration as determined in cases with normal adrenal cortical function is described The most characteristic feature found during the administration of ACTH is an

increase in steroid excretion which takes place as early as the first day. This continues *progressively* until a maximum is reached on the 3th—5th day. As well as being dependent on the dosage, the maximum response is subject to rather large individual variations.

b. In 4 cases a reduced adrenal cortical function is found and the results of the steroid analyses are compared with those obtained from a 48-hour ACTH test performed on a woman with Addison's disease. The urinary steroids increase slowly and irregularly and no distinct maximum is seen. The slow reaction continues after the discontinuation of the ACTH treatment, and the excretion values only reach the pre-treatment level in the course of several days. In some of the cases only the corticoids showed these characteristic changes. There seems therefore to be good reason for assessing these steroids in addition to the 17-ketosteroids in Thorn's 48-hour test for the evaluation of the adrenal cortical function.

2) In 2 cases treated with varying doses of ACTH the average increase in the urinary steroids with different dose levels was calculated. When these values for excretion were plotted against the logarithm of the dose a linear relationship was found. The comparison of these curves with the results of the other experiments shows that in order to produce an increase in corticoid excretion in children a minimum effective dose of 5—6 mg. ACTH/24 hours must be administered In adults this is about 12 mg., while the corresponding figures in the case of 17-ketosteroids are about 12 and 14 mg./24 hours respectively. There is evidence that the doses mentioned correspond to the amounts of ACTH actually elaborated by the hypophysis

3) In 3 cases treated with high doses of ACTH a decrease in corticoid excretion is seen after 9 to 10 days and in one of the cases, a decrease in the 17-ketosteroid excretion. The possibility of exhaustion is discussed, and it is pointed out that the adrenal cortex may become depleted of corticoids when the maximum dose is administered in a single injection In adults, this maximum dose is apparently between 20 and 30 mg.

4) In most of the cases a greater percentage increase is

measured by the urinary steroids. It should be stressed that the eosinophil response is a secondary effect of ACTH and that if it fails to appear this does not necessary mean a decreased response of the adrenal cortex to ACTH but may be due to an altered reactivity of the »end organs« to the corticoids.

In those cases referred to in which only a few steroid analyses have been made during treatment with ACTH no particular information has been obtained except that it can be ascertained whether the response of the adrenal cortex is constant or changes gradually. This is of special interest in long-term experiments. In the case of high doses of ACTH in short-term experiments it should be of interest to investigate the steroid excretion throughout the whole period of treatment. In such experiments the method of study used in case 1 and 2 could be used in the future, and an overwhelming large number of analyses thus avoided. In these cases the urine was collected over periods of 3 days before, and during the treatment. Daily analyses were performed on the first and second day and from the data thus obtained the functional capacity of adrenal cortex could readily be ascertained. The potential functional reserve, as measured by the maximum increase during the following period of treatment, cannot, as is seen in the two cases, always be assessed by the analyses carried out in the two first days.

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found in corticoid excretion than in the 17-ketosteroids. This is clear as early as the first day of ACTH treatment. This difference in the excretion rate of the two groups of steroids, under normal conditions, is particular marked in infants as compared with adults. During ACTH administration to 2 children, the excretion of corticoids increased in a manner similar to that found in adults, while the 17-ketosteroids increased only slightly. In one of the cases a commencing »maturity« of the androgenic function of the adrenal cortex was seen after 50 days of ACTH treatment. The results indicate that corticoids and 17-ketosteroids are produced independently of each other in the adrenal cortex The androgenic function (or ability to elaborate 17-ketosteroids or their precursors) is only fully developed at the time of puberty.

5) In long-term experiments with ACTH a refractory state was observed in 2 cases after 70 and 40 days of treatment respectively after which time no increase in the steroid excretion was seen even when the dose of ACTH was increased. After 50 days' treatment of a child, a decreased corticoid response was seen together with a normal or slightly increased 17-ketosteroid response. The possibility of antibody (antihormone?) formation is discussed.

ACKNOWLEDGEMENT

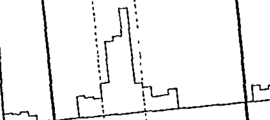
I am greatly indebted to the National Danish Association against Rheumatic Diseases for generous support without which this extensive study could not have been carried out.

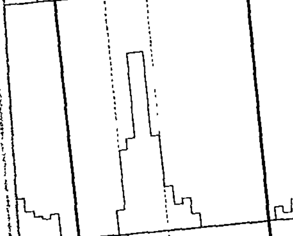
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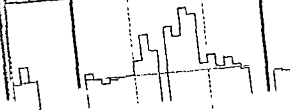
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From the Hormone Department of the State Serum Institute, Copenhagen.

INVESTIGATIONS ON THE BIOLOGICAL DETERMINATION OF GLUCOCORTICOIDS

BY

MOGENS SPRECHLER

In 1942 *Reinecke* and *Kendall* worked out a method for the biological determination of the so-called glucocorticoids which are produced by the adrenal cortex. Subsequently several modifications appeared. All of these methods are in principle based on the ability of the glucocorticoids to deposit glycogen in the liver of the adrenalectomized rat or mouse. As previously mentioned (*Sprechler*, 1949), the method of *Venning, Kazmin & Bell* (1946) seems to be sufficiently sensitive for the determination of the small amounts of glucocorticoids excreted in the urine of normal man. As it was intended in the present study to include methods for the determination of the urinary corticoids, the method of Venning et al was examined at the end of 1947.

Method. The procedure devised by the authors has been strictly followed and, only a short description will therefore be given here.

As test animals, male white mice from our own colony were used. The weight ranged from 20 to 25 gm., in most cases just above 20 gm. The animals were anesthetized with ether and adrenalectomized by the lumbar route Afterwards they were placed in a box at a constant temperature: 28° C. ± 1° and kept on a constant diet containing: 600 gm. casein, 1200 gm baked wheat starch, 8 gm. sodium

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chloride, 8 gm calcium carbonate, 2 gm. ferric citrate, 2 gm. sodium iodide, 200 gm. F. yeast and 20 gm. cod liver oil. On the first post-operative day 0.9 per cent NaCl containing 5 per cent glucose was given as drinking water, and on the following days 0.9 per cent saline only. The food is removed on the afternoon of the third post-operative day. The following morning the drinking water is also removed and the mice transferred to clean cages From 9 00 a m to 2.15 p. m. the material to be tested is given subcutaneously in 7 equal doses, the first three are given at intervals of 45 minutes and the remaining four at hourly intervals One hour after the last injection the mice are killed and within 30 seconds the liver is removed and immediately frozen between two plates of dry ice. After weighing, the livers are dropped into centrifuge tubes containing 2 ml of 30 per cent KOH which have previously been placed in a boiling water bath The tubes are shaken several times for the next 30 minutes after which all the tissues is dissolved After cooling, the glycogen is precipitated by adding ½ ml. of 1 per cent sodium sulphate solution and 6 ml of 95 per cent alcohol and stirring The next morning the tubes are centrifuged for 20 minutes. The supernatant liquid is removed by suction and the precipitate is dissolved in 1 ml. of distilled water, precipitated by means of sodium sulphate and alcohol, heated on a water bath to boiling point, and then cooled and centrifuged. This washing and precipitation is repeated once more but this time without the sodium sulphate. After drying overnight, the glycogen is dissolved in 2 ml of distilled water and 2 ml 10 N H_2SO_4 are added, after which the hydrolysis is carried out by heating on a boiling water bath for 30 minutes After cooling and neutralization of the solution the glucose is determined by the method of *Schaffer & Somogyi* (1933) and *Somogyi* (1937, 1945). The glycogen content is *then obtained by multiplication with the factor 0 927 and the result* calculated as mg liver glycogen per 100 gm body weight

Preparations of extracts.

The day before the assay, the test material, i. e. steroid or urinary extract, is dissolved in absolute alcohol in such an amount that the final solution contains 10 per cent of alcohol. The total dose administered to each mouse is always given in 1.4 ml. (0.2 ml. in each injection). In this volume is also included the required amount of glucose which is referred to below.

Urinary extracts. In the case of a normal content of glucocorticoids the 24-hour or 48-hour urine can be used, but in

these case of a low titer, several 24-hour urines may be necessary if more exact values are required. The urine is acidified to pH 1 with 40 vol. per cent H_2SO_4 (measured with a glass electrode) and immediately extracted by shaking vigorously three times with 25 per cent (by volume) of chloroform in a separating funnel. Emulsions are broken by centrifuging. If the combined chloroform extracts exceed 300 ml. this is evaporated to a lower volume before proceeding with the washings. Otherwise it is immediately washed with n/10 NaOH. 10—20 ml. 3 times, and with distilled water, 10—20 ml. twice Each washing is extracted back with the same volume of chloroform which is added to the combined extract before going on to the next washing. Afterwards the extract is evaporated to 1—2 ml. in a vacuum distillation apparatus at a temperature not exceeding 45° C. Finally the extract is transferred to a small test tube with small amounts of chloroform and evaporated to dryness. The dry residue can be stored in a refrigerator for a long time without any loss of biological activity.

Effect of glucose on the deposition of liver glycogen in the fasted adrenalectomized mouse.

Venning et al. (1946) obtained a high sensitivity in their assay by the administration of glucose along with the glucocorticoids. They found that a maximum dose of 70 mg. glucose could be given without causing any significant increase of the liver glycogen level.

Several control assays with glucose were carried out in this laboratory partly in order to determine the size of the maximum dose and also to ensure that no alteration in the sensitivity of the animals occurred from time to time. For a long period 70 mg. of glucose was found to be a suitable dose. The few high values for the liver glycogen found especially at the beginning of the study were in most cases explained by the fact that remnants of adrenal tissue persisted and were found by macroscopic examination after the assay. During the last

chloride, 8 gm. calcium carbonate, 2 gm ferric citrate, 2 gm. sodium iodide, 200 gm. F. yeast and 20 gm. cod liver oil. On the first postoperative day 0.9 per cent NaCl containing 5 per cent glucose was given as drinking water, and on the following days 0.9 per cent saline only. The food is removed on the afternoon of the third postoperative day. The following morning the drinking water is also removed and the mice transferred to clean cages. From 9.00 a. m. to 2:15 p. m. the material to be tested is given subcutaneously in 7 equal doses, the first three are given at intervals of 45 minutes and the remaining four at hourly intervals. One hour after the last injection the mice are killed and within 30 seconds the liver is removed and immediately frozen between two plates of dry ice. After weighing, the livers are dropped into centrifuge tubes containing 2 ml. of 30 per cent KOH which have previously been placed in a boiling water bath. The tubes are shaken several times for the next 30 minutes after which all the tissues is dissolved. After cooling, the glycogen is precipitated by adding ½ ml. of 1 per cent sodium sulphate solution and 6 ml. of 95 per cent alcohol and stirring. The next morning the tubes are centrifuged for 20 minutes. The supernatant liquid is removed by suction and the precipitate is dissolved in 1 ml. of distilled water, precipitated by means of sodium sulphate and alcohol, heated on a water bath to boiling point, and then cooled and centrifuged. This washing and precipitation is repeated once more but this time without the sodium sulphate. After drying overnight, the glycogen is dissolved in 2 ml. of distilled water and 2 ml. 10 N H_2SO_4 are added, after which the hydrolysis is carried out by heating on a boiling water bath for 30 minutes. After cooling and neutralization of the solution the glucose is determined by the method of *Schaffer & Somogyi* (1933) and *Somogyi* (1937, 1945). The glycogen content is then obtained by multiplication with the factor 0.927 and the result calculated as mg. liver glycogen per 100 gm. body weight.

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Urinary extracts. In the case of a normal content of glucocorticoids the 24-hour or 48-hour urine can be used, but in

In order to determine the effect of glucose administration together with the glucocorticoids on the sensitivity of the assay, the following experiments were carried out: 4 groups of fasted adrenalectomized mice (each group including 8 animals) were given 0, 20, 40 and 60 mg. glucose, respectively and in addition all of them received 40 µg 17-hydroxy-11-dehydrocorticosterone (Cortisone, Cp. E.). The average values for the liver glycogen were found to be: 2.1, 1.6, 27.3 and 36.0 mg. per 100 gm. body weight respectively. In another experiment 40, 60 and 80 µg of glucocorticoids was administered, but no glucose. The liver glycogen deposition was 1.9, 1.6 and 2.1; some of the animals died and all of them were in a very poor condition during the assay.

Relationship between dose of Cortisone, »Cortine« and urinary extracts on the liver glycogen deposition.

As a standard of reference Cortisone was chosen. The results of standardization with this steroid are listed in Table 1. Experiments were carried out on three different days with doses of 20, 40, 60 and 80 µg. The individual values for the liver glycogen are illustrated in Fig. 2. Although great variation in these values was observed, a linear relationship was found between the logarithm of the dose and the mean values of the response.

In a few experiments carried out with the acetate of Cortisone at the same dose levels (calculated on the basis of the molecular weight) we have found that the response is somewhat lower at the high dose levels (40 and 80 µg). This phenomenon may be due to the very poor solubility of this substance causing a delayed absorption in these short-term experiments.

Table 1 also contains the results obtained in experiments with an adrenal cortical extract (»Cortine«, Organon) and urinary extracts obtained from two patients before (MP-I and BJ-I) and during the administration of adrenocorticotrophic hormone (ACTH) (MP-II and BJ-II). The calculated regression lines for the extracts are shown in Fig. 3 compared with

few months, however, a new stock of mice has been used, and in a few control assays there appears to be a slight increase in the liver glycogen level, suggesting that the dose of glucose is now too high.

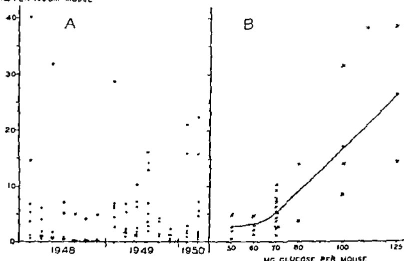


Fig 1

A Effect of glucose administration on liver glycogen in fasted adrenalectomized mice (control assays on 98 animals) The dose of glucose is 70 mg The dots represent the individual response. *B:* Effect of a dose of glucose on the liver glycogen. Each cross represent the average result of an assay on 4—10 animals.

In Fig. 1 A the results of 15 control assays on (98 mice) treated with 70 mg. of glucose are shown. The dots represent the individual liver glycogen values. Apart from the above mentioned high values the variation is only within a narrow range. The results of assays with various doses of glucose are shown in Fig. 1 B Each cross here represents the mean liver glycogen deposition in an assay performed on 4—10 animals and the circles indicate the mean of all the assays. A dose response curve can be drawn and it is clearly seen that increasing doses of glucose above 70 mg. result in a steep increase in the deposition of liver glycogen.

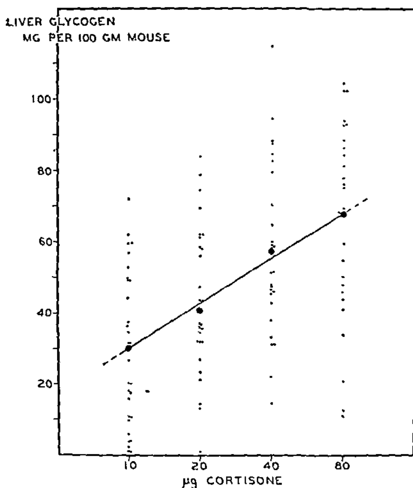


Fig. 2.
Logarithmic dose response curve for 17-hydroxy 11-dehydrocorticosterone The small dots represents the individual liver glycogen values

the standard curve for Cortisone. The agreement between the log. dose-response relationship for Cortisone, »Cortine« and urinary extracts MP-I and BJ-I is clearly indicated by the fact that the curves are parallel. The curves for the urinary extracts MP-II and BJ-II (from the period of ACTH administration) are undoubtedly somewhat steeper

Table 1.

Compound	Dose	Number of mice	Mean liver glycogen	Standard deviation
	µg		mg/100 g mouse	
17-hydroxy-11-dehydrocorticosterone				
	10	10	23.8	18.8
	10	10	27.6 (30.0)	19.5 (20.1)
	10	10	38.6	23.7
	20	10	48.6	19.5
	20	10	31.7 (40.9)	23.7 (21.1)
	20	10	42.4	22.3
	40	10	61.6	23.1
	40	10	56.8 (57.4)	18.2 (21.8)
	40	10	53.8	25.2
	80	10	76.3	27.9
	80	10	57.5 (68.0)	23.6 (25.0)
	80	10	70.1	25.8
Cortine	ml			
	0.25	6	36.8	21.7
	0.50	8	50.8	28.7
	1.00	8	64.9	26.2
Urinary extracts	Hours' urine			
MP-I	3	8	11.1	6.5
	6	12	17.3	9.0
	12	8	29.7	20.9
MP-II	1/8	12	18.2	14.8
	1/4	12	52.7	20.4
	1/2	12	51.3	24.9
	1	13	79.5	29.3
	2	13	80.2	34.6
BJ-I	3	8	15.8	12.6
	6	8	46.2	14.5
	12	6	39.6	25.5
BJ-II	1/2	11	29.5	22.8
	1	11	42.0	19.5
	2	11	67.7	21.2

Liver glycogen deposition in fasted adrenalectomized mice treated with Cortisone, Cortine and extracts of urine from 2 patients: MP-I and BJ-I before, MP-II and BJ-II during the administration of ACTH. The standard deviation is calculated from the formula

$$s = \pm \sqrt{\frac{\sum d^2}{n-1}}$$

specificity of the method. Experiments have been performed on 7 cases in whom the diagnosis was clear from the clinical course of the disease. The results are listed in Table 2. Because of the very low excretion it is often necessary to make up the extract from several pooled 24-hour urine specimens. Nevertheless, in several assays it has been impossible to give an exact figure for the low 24-hour excretion when this was below 10 µg. In several of the assays the liver glycogen did not exceed the control level, indicating a zero value for the excretion. In one case of hypo-pituitarism very low excretion values were also found in the three analyses

A correlation between the excretion of glucocorticoids and the pathological findings was established in a case of Cushing's syndrome due to carcinoma of the adrenal cortex. Before the operation the excretion was 1909 µg per 24 hours, and one month after the operation 354 and 254 µg per 24 hours were found on two different days. The corresponding values for 17-ketosteroids were 207 and 45 8 mg./24 hrs respectively. Normal values were not obtained. Shortly after the last analysis the patient died and metastases were found in the liver and bones. In all probability these were responsible for the increased production of corticoids post-operatively.

Determinations of the excretion of glucocorticoids in the urine of normal subjects were performed in 16 cases: 2 women and 14 men. The results are given in Table 2 In cases 3, 5 and 9, determinations were made on two, and in case 8, on 4 different 24-hour urine specimens A rather large variation is seen. The average excretion in the 14 men was 64 µg per 24 hours and in the two women 35 µg

For a further elucidation of the daily variations of the excretion some of the results obtained in a patient previously described (*Brochner-Mortensen et al.*, 1949) will be mentioned. The patient received ACTH treatment and the determination of the glucocorticoid excretion was continued for a period of 22 days following the cessation of the treatment Each extract was made from samples of urine obtained on consecutive 48-hour periods The values (Table 2) ranged

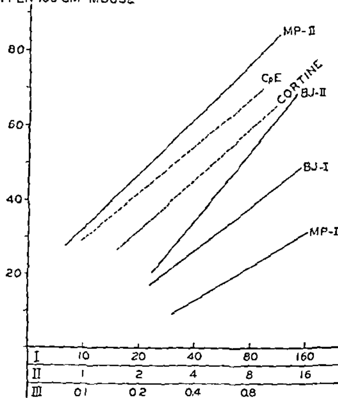


Fig 3

Logarithmic dose-response regression lines for Cortisone, Cortine and urinary extracts MP-I and BJ-I (before ACTH) and MP-II and BJ-II (during ACTH treatment). Note the three scales of the abscissa: I µg Cortisone, minutes' urine equivalent of MP-II and BJ-II. — II: Hours' urine equivalent of MP-I and BJ-I — III ml Cortine

Application of the method in single determinations of urinary glucocorticoid excretion.

Using the method of assay described above, *Venning & Browne* (1947) found very low values for the excretion of glucocorticoids in cases of Addison's disease. Urinary extracts from such patients seem to be very suitable for evaluating the

DISCUSSION

When previous fasting results in a depletion of liver glycogen in the adrenalectomized animal, the glucocorticoids cause a deposition of glycogen which can be measured by this method as well as by the original method of *Reinecke & Kendall*. The results presented in this paper confirm the statements of *Venning et al.* on the decisive importance of glucose administration in the sensitivity in such assays performed on fasted adrenalectomized mice. An increase in liver glycogen can, however, be obtained when high doses of glucose are administered alone and it is, therefore, very important to carry out control assays at various intervals with the dose chosen. The authors mentioned above found a seasonal variation, as their standard dose of glucose caused a slight increase of the liver glycogen level during the summer months. This has not been observed in our laboratory.

The variation in the individual response seems to be somewhat larger than found by *Venning et al.* and the slope of regression lines for Cortisone and urinary extracts is undoubtedly less steep. These discrepancies can, perhaps, be ascribed to differences in the test animals used.

On comparing the log dose-response curves for Cortisone, »Cortine« and the two urinary extracts: MP-I and BJ-I a very striking similarity is seen, which justifies the use of Cortisone as a standard of reference in these cases. With regard to the results of the assays with the urinary extracts: MP-II and BJ-II, however, some interesting problems arise. There is no doubt as to the steeper course of the log dose-response curves for these two extracts, which seems to indicate a qualitative alteration of the active material excreted in the urine during the period of ACTH administration. As previously suggested (*Sprechler*, 1950), this possibility would explain some of the discrepancies observed between the chemical and biological values obtained for the excretion of corticoids during ACTH administration. The glucocorticoids in the cases referred to were calculated from the standard curve for Cortisone

The idea of such qualitative alterations in the composition

from 25 to 89 µg per 24 hours with an average of 48 µg/24 hours.

Table 2.

Normal					Daily variations in L.R. (woman, age 53)		Addison's disease				
Sex	Age	No		Excretion µg/24 hrs.	Date 1949	Excretion µg/24 hrs	Patient	Sex	Age	Date	Excretion µg/24 hrs
					July						
Males	19	16		46	11—12	36	H C	M	26	19/2 48	0
	22	14		113	13—14	53				12/3 -	0
	27	13		51	15—16	67				5/5 -	0
	28	11		77	17—18	32				8/9 -	0(<10)
	31	5a	48		19—20	÷				21/12 -	0(<10)
		5b	113	81	21—22	64					
	35	9a	29		23—24	57	K.H.	F	53	13/3 -	<10
		9b	36	33	25—27	89				9/4 -	0
	36	7		42	28—29	25	R L.P.	F	34	23/3 -	0
	37	2		108	30—31	25	F.M.	F	44	9/7 -	0
	39	4		35	August		S B.	F	27	9/8 -	0
	41	12		56	1—2	30					
	42	3a	33		Average	48	H.H	M	44	23/8 -	33
		3b	83	58						17/9 -	26
	42	10		48						1/10 -	<10
	43	1		77						4/11 -	<10
	46	15		69						2/12 -	<10
		Average:		64						12/3 49	<10
Females	19	8		30							
	26	10a	25				A.N.	F	51	4/1 49	15
		b	46				Hypopituitarism				
		c	61	40			A L	F	32	9/9 48	<20
		d	28							11/9 -	<20
		Average		35						9/10 -	24

Excretion of glucocorticoids in 16 normal subjects (2 women and 14 men) Col I. — Excretion of glucocorticoids in 11 consecutive 48-hour urine specimens from a woman (age 53, suffering from chronic rheumatoid arthritis). Col II. — Excretion of glucocorticoids in 7 patients with Addison's disease and 1 with hypo-pituitarism. Col. III.

The results of assays carried out with extracts of urine from normal persons agree very closely with those found by *Venning & Kazmin* (1946) but a greater variation in the values was observed.

SUMMARY

The liver glycogen deposition method of *Venning, Kazmin & Bell* (1946) for the biological assay of the glucocorticoids is reexamined and is found to be suitable for the quantitative determination of the urinary excretion of these substances.

Experiments with urinary extracts collected from patients before and during the administration of ACTH seem to indicate a qualitative alteration in the composition of the corticoids during the period of stimulation. This may explain some of the discrepancies found between the values of the chemical and biological determinations of corticoids previously described with ACTH treatment. When calculating the results Cortisone is suitable as a standard of reference in cases of normal function of the adrenal cortex, while the use of Cp. F. seems more suitable in cases of hyperfunction of the gland.

Very low excretion values were found in 7 cases of Addison's disease and in 1 case of hypo-pituitarism due to a hypothalamic lesion.

The urinary excretion of glucocorticoids was assayed in 2 normal women and 14 normal men. The average excretion was 35 µg and 64 µg per 24 hours respectively. Consecutive 48-hours urine collections from a woman (with chronic rheumatoid arthritis) were assayed for 22 days. The excretion ranged between 25—89 µg/24 hrs. with an average of 48 µg/24 hrs.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr *J Heer* and Dr. *A Wettstein* Ciba, Basle, for the preparation of the free crystalline Cortisone from the acetate, and to Dr. *Frederik Paulsen*, Organon, Stockholm, for a »Cortine« preparation

of the biologically active corticoids in the urine following stimulation of the adrenal cortex also seems to be supported by the findings of *Mason* (1950). From extracts of urine obtained from patients treated with ACTH, he was able to isolate large amounts of 17-hydroxycorticosterone (Cp. F) while Cortisone could not be detected at all. On the other hand, *Schneider* (1950) could only isolate Cortisone from a normal male urine but not Cp. F. Finally it is of great interest to recall the investigations of *Olson et al.* (1944). They showed that Cp. F. produced a response in the liver glycogen deposition which differed qualitatively from that of Cortisone and from that of several adrenal cortical extracts, and pointed out that the slope of the regression line which was steeper in the case of Cp. F. should be a good criterion of these qualitative differences.

Unfortunately we have up to the present not been able to obtain Cp. F. Comparative studies with this steroid and Cortisone and further experiments with differents urinary extracts are necessary for the final solution of these problems.

It may be concluded that in the case of a normal functional adrenal cortex (and probably also in the case of hypo-functional states) Cortisone is suitable as a standard of reference in the assay of urinary excretion of glucocorticoids, while the use of Cp. F. appear to be more suitable in cases of hyperactivity of the adrenal cortex.

The results of assays with extracts of urine from patients with Addison's disease indicate that the method has a very high specificity. In a few cases a slight activity was found and this agrees with the results obtained by the chemical determination of urinary corticoids (*Sprechler*, 1950). As summarized by *Venning & Browne* (1947), we do not, as yet, know if the impairment of the adrenal cortex involves the glucocorticoid production to the same extent in all cases, and the clinical signs of insufficiency probably appear at an early stage of the disease at which only slight pathological changes are present in the adrenal cortex. This suggestion seems to be supported by the findings obtained in case *H. C.* (*Table 2*).

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By means of biological assays (based on the ability of certain steroids (glucocorticoids) to deposit glycogen in the liver of the fasted adrenalectomized mouse) *Venning & Kazmin* (1946) found that the urinary excretion of corticoids in 14 normal men (aged 20—51 years) ranged between 40 and 85 µg/24hrs. and in 14 normal women (aged 21—45 years) between 25 and 65 µg/24 hrs. The average excretion amounted to 60 and 41 µg/24 hrs. respectively. In a determination of the excretion in a few children the values were found to reach the adult level at the age of 5½—7 years. *Venning, Randall & Gyorgy* (1949) examined 19 newborn children and found excretion values ranging between 4 and 17 µg/24 hrs and on an average an amount equivalent to 11 µg. Cortisone per 24 hours.

Using the technique given by the above mentioned authors, excretion values of a similar range were found here (*Sprechler*, 1951). 14 normal men and 2 normal women excreted 33—133 µg/24 hrs. (average, 64) and 30—40 µg/24 hrs respectively.

Only these two reports involving the examination of a small number of subjects are concerned with a quantitative determination of the biologically active glucocorticoids excreted by the normal subject. During the last few years, however, several publications on the normal excretion of corticoids as determined chemically have appeared It should be pointed out that the absolute values obtained in this way are considerably higher than those found in the bioassays, and this is, in all probability, due to the fact that some of the biologically inactive adrenal cortical metabolites are included in the chemical determination. Different methods have been used in these studies and the results mentioned below cannot, therefore, be directly compared.

As previously mentioned (*Sprechler*, 1950 a) two main groups of methods have, up to the present, been suggested for the chemical determination of corticoids in urine. Both are based on the special properties of the characteristic side chain attached at carbon atom no. 17 in the molecule of the corticoids. One of these groups is based on the reducing property

Separatum Acta endocrinologica 7, 330—367, 1951.

From the Hormone Department of the State Serum
Institute, Copenhagen.

INVESTIGATIONS ON THE NORMAL EXCRETION OF CORTICOIDS IN THE URINE OF MAN AND THE RELATION OF THE CORTICOIDS TO THE 17-KETOSTEROIDS

BY

MOGENS SPRECHLER

PREVIOUS INVESTIGATIONS

Since 1931 it has been known that in normal man substances are excreted in the urine which exert a biological effect identical to that of adrenal cortical extract and of several crystalline steroid hormones isolated fra the adrenal cortex.

For several years the determination of these substances could be performed only by means of biological tests. Only a few of these methods were, however, sensitive enough for an exact quantitative titration of the small amounts of active substances present in normal urine. A great advance has been made during the last few years by the introduction of different chemical methods for the determination of the corticoids and their metabolites By means of these methods it is possible to obtain a more rapid and presumably a more exact determination.

A review on these fundamental investigations has previously been given (*Sprechler*, 1949) Further investigations on the adrenal cortical function in normal man has subsequently been reported in the literature and hence the main points are summarized here.

Table 1.
Studies on the normal excretion of corticoids previously reported in literature.

	pH of urine	Benzene-water partition*	Girard's process	Number of subjects	Sex	Age	Excretion of Corticoids mg/24 hrs. Range	Average
Reduction-methods								
Heard, Sobel & Venning (1946) (P)	1	–	—	9	F	adults	1 0—2 0	1 34
				9	M	»	1 1—2 1	1 53
				4	M	2—>7 yrs	0 32—>0 79	
Matson & Longwell (1949) (P)	1	—	—	13	M	newborn	0 17—0 62	
				?	?	?	1 04—2 54	
Talbot, Albright et al (1947) (Cu)	untreated urine	+	+	8	F	adults	0.10 – 0 44	0 22
		+	+	12	M	»		
Talbot (1949) (Cu)	»	+	+	80	F+M	0—18 yrs	(0 03—0.25§)	
				28	»	adults		
Day (1948) (Cu)	»	+	+	7	?	newborn	0 00—0 20	0 07
Formaldehyde-methods								
Lowenstein, Corcoran & Page (1946)	»	—	—	?	M	adults	0 50—0 80	
Daughaday, Jaffe & Williams (1948)	1 7	+	—	3	F	»	1 0—1 6	
				6	M	»		
Corcoran & Page (1948)	1 0	—	—	10	F	»	0 28—1 63	0 84
				10	M	»	0 72—1 66	1 15
Tobian (1949)	1 0	—	—	10	F	»	0 54—1 32	0 98
King & Mason (1950)	1 0	—	—	63	F	0->15 yrs	0 09—>0 85	0 18—>0 45
				32	M	»		
				12	F	adults	0 44—0 79	0 60
				19	M	»	0 31—1 16	0 52
Read, Venning & Ripstein (1950)	?	+?	—	10	M	newborn	0 025—0 133	

P Phosphomolybdic-acid-reduction Cu: Copper-reduction
* Determinations carried out with water-soluble steroids
§ The excretion expressed in mg per sq m. of body surface

of the side chain for the cupric ion (Talbot et al., 1945) and for phosphomolybdic acid (*Heard & Sobel*, 1946). The second group is based on the formaldehyde liberating property of the side chain for periodic acid oxidation.

Furthermore the methods differ from each other by a different treatment of the urine (1): chloroform extraction of unacidified urine, 2): extraction immediately after acidification of urine to pH 1,7 or 1,0, 3): extraction after 1—3 days' *mild hydrolysis) and of urine extract (chemical determination 1)*: directly on the total crude extract, 2): on the water-soluble substances obtained from a benzene-water partition of the crude extract, 3): *on the ketonic substances isolated from the extract after treatment with Girard's reagent T)*.

For the sake of clarity the values for the excretion of corticoids in normal human beings reported in the literature are outlined in Table 1. As can be seen rather comprehensive investigations have been carried out with *children. The values in the newly-born infant are low but afterwards the excretion rises gradually during childhood. In particular this was clearly* demonstrated by *King & Mason* (1950) who examined 95 children and found an increase amounting to 0 01 mg. for each yearly increase in age. In a study of 80 children and 28 adults *Talbot* (1949) calculated the excretion values per square meter of body surface and claimed that the excretion expressed in this way is constant during life. The absolute values for the excretion were not given.

The investigations of the normal excretion of corticoids in adults have been performed in only a small number of subjects. The results vary a great deal Some investigators (*Heard, Sobel & Venning*, 1946, *Corcoran & Page*, 1948) found that the excretion in men was higher than that in women, while others did not observe such a difference Work reported by *Romanoff, Plager & Pincus* (1949) on 34 men and 34 women with an age ranging from *20 to 80 years is more difficult to evaluate as* the determinations were carried out on three consecutive 8-hour urine specimens in each individual case instead of using the 24-hour collection of urine. *The corticoids were determined*

average excretion curve for women from that obtained for men he found a curve quite similar to that previously established for the excretion of biologically active androgenic substances during the life of normal men (*Hamburger, Halvorsen & Pedersen*, 1945).

In the case of men the above mentioned results were shortly afterwords confirmed by *Kirk* (1949), who examined the excretion in 77 men (age, 40—97 years) and by *Kenigsberg, Pearson & McGavack* (1949), whose material comprised 85 men (age, 13—75 years) and 20 women (17—61 years) and a few children.

Some further details concerning these investigations will be discussed lateron together with the results obtained in the present study.

OWN INVESTIGATIONS

. *The aim* of the present study has been in the first place to establish the limits for the normal excretion of corticoids in the urine by an examination of the excretion in a large number of individuals of both sexes and of all age-groups. This appeared necessary as the method used in this laboratory has not previously been used in such an examination. Furthermore, the purpose was to examine the daily individual variations in the excretion and at the same time to determine particularly whether any relation existed between the excretion and the menstrual cycle in women.

By a simultaneous determination of the 17-ketosteroids it was possible to compare the excretion patterns of the two groups of steroids.

Material and technique. As normal subjects individuals were chosen with no previous history of any serious disease or any endocrine disorder They were in good health just before and during the period of experiment. The material comprises 214 females between 2 and 83 years of age and 187 males between 2 and 80 years of age. In all these cases the analyses were carried out on a single 24-hour urine specimen

by the use of both groups of chemical methods previously mentioned. A significant diurnal variation in the excretion was found in men, as the excretion was higher in the forenoon than in the afternoon and the lowest values were found in the night specimen. In women the diurnal variation was less marked. No variation with age was observed just as no significant sex difference can be deduced from the results.

As can be seen various problems concerning the normal excretion of corticoids in urine, particularly in adults, seem as yet to be unsolved. Much more experience has been gained on another group of steroids, the 17-ketosteroids, normally excreted in the urine. In woman, these steroids for the most part, or perhaps exclusively, originate from the adrenal cortex but in man they are in addition metabolites from the testis hormone. During the first few years following the reports of *Zimmermann* (1935) and of *Callow, Callow & Emmens* (1938) on their fundamental investigations on the chemical determination of 17-ketosteroids, numerous publications appeared on the normal excretion in urine. It would be outside the scope of this work to mention all these reports in detail, especially as they were all concerned with a small number of individuals and offered a very incomplete picture of the actual conditions. In 1948, however, three large investigations were reported at the same time and independently of each other: *Hamburger* (1948) examined 137 men (age, 3—102 years) and 127 women (age, 2—92 years), *Robinson* (1948) examined 128 men (age, 0—90 years), and *Hamilton & Hamilton* (1948) examined 51 men (age, 21—75 years). With the appearance of these investigations the normal excretion during all age-groups was charted. Very low values are found during the first years of childhood followed by a steep increase in the excretion at the age of about 12 years This increase continues until a maximum is reached between 20 and 25 years after which age the excretion gradually decreases reaching low values in old age.

Only the investigation of *Hamburger* also dealt with a large number of women. As would be expected, the excretion is somewhat lower in women than in men. By subtracting the

RESULTS

Dependence of the corticoid excretion on age and sex.

The total data of the corticoid analyses are presented graphically in Fig. 1. Milligrams of corticoids are plotted against age in years and each dot represents an individual ob-

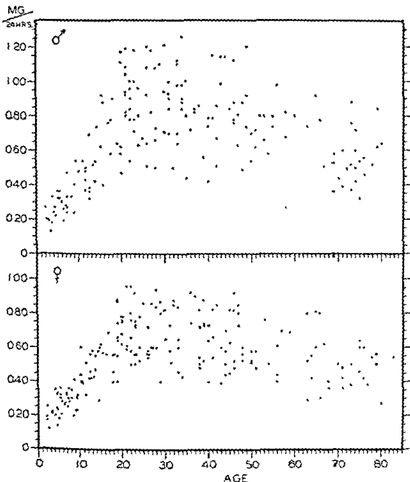


Fig 1
The 24-hour excretion of corticoids in the urine of 214 normal women and 185 normal men Each dot represents the excretion in a single person. Ordinate. mg per 24-hours Abscissa chronological age

It was not possible to obtain urine from the same number of individuals in the various age groups. Especially in the case of women aged between 50 and 65 years only a relatively small number of subjects were examined. The distribution of the total number of individuals examined according to age appears clearly from the following diagrams.

Apart from a few cases the nett body weight and height were obtained. The body surface area in each individual was calculated by means of the formula devised by Du Bois: S. B. A. = $H^{0.725} \times W^{0.425} \times 71.84$, in which H = height and W = body weight, and the body surface area is expressed as square meters.

After a thorough instruction, the urine was collected without preservative during exactly 24 hours and quickly afterwords placed in the cold. In a single case no determination of the corticoids was obtained because the urine was infected and the emulsion formed with chloroform could not be broken.

The corticoid determination. The extraction of corticoids was, as a rule, carried out within 24 hours following the termination of the urine collection. The volume of urine used for the analysis was in the case of small children $^1/_5$ and in all other cases $^1/_{10}$ of the 24-hour specimen The urine was acidified to pH 1 with 40 vol per cent of H_2SO_4 and immediately extracted by shaking 3 times with 25 per cent (by volume) of chloroform. As previously described (*Sprechler*, 1950 a) the chloroform extract is then extracted with NaOH and washed with water Following evaporation to dryness the residue is treated with Girard's reagent T. The reducing power of the total neutral ketonic fraction is finally determined with phosphomolybdic acid reagent As a standard of reference desoxycorticosterone was used.

The 17-ketosteroid determination was always carried out by the micromethod of *Hamburger & Rasch* (1948). The volumes of urine were here $^1/_{25}$ and $^1/_{50}$ of the total 24-hour specimen A correction for unspecific chromogenic substances was as usual made. In a few cases no determination of the 17-ketosteroids was obtained because of the loss of the urine.

Table 2.

The excretion of corticoids and 17-ketosteroids in normal subjects of various age-groups

Age Group	Women				Men				Corticoids μg/24 hrs				17-Ketosteroids μg/24 hrs			
	Average Age	Number	Corticoids mg/24 hrs	17-ks mg/24 hrs	Average Age	Number	Corticoids mg/24 hrs	17-ks mg/24 hrs	per sq m body surface		per kilogram of B W		per sq m body surface		per kilogram of B W.	
									♀	♂	♀	♂	♀	♂	♀	♂
0—4	3.5	11	0.224	0.83	3.2	12	0.228	0.67	344	352	14.4	14.6	1060	910	54.5	42.9
5—9	7.1	25	0.285	1.32	7.1	17	0.321	1.58	320	362	12.3	14.0	1310	1830	50.2	60.6
10—14	12.5	17	0.472	2.81	12.4	15	0.475	2.87	362	358	12.1	11.8	2180	2080	71.0	68.3
15—19	17.8	15	0.610	7.44	16.8	9	0.720	7.60	376	426	10.7	12.2	4600	4380	132	123
0—9	5.8	39	0.263	1.14	5.5	29	0.282	1.19	329	358	13.1	14.3	1210	1430	52.2	58.6
10—19	14.9	32	0.536	4.98	14.0	24	0.566	4.72	369	390	11.5	12.0	3310	3060	99.7	91.8
20—29	24.0	42	0.694	8.34	23.6	38	0.866	13.10	423	467	11.9	12.4	5160	7090	146	180
30—39	35.1	23	0.694	6.80	33.5	27	0.858	11.40	421	466	11.4	12.4	4180	6190	114	164
40—49	44.2	28	0.631	5.55	44.5	23	0.836	10.40	370	465	9.9	12.2	3210	5720	84.4	150
50—59	53.3	13	0.567	4.74	53.5	13	0.726	8.28	338	373	9.0	9.2	2800	4270	73.5	105
60—69	65.5	13	0.542	3.46	66.0	8	0.653	6.90	317	360	7.7	9.0	2020	3820	48.2	86.9
70—79	74.0	22	0.490	3.50	74.1	21	0.550	4.50	295	300	7.4	7.4	2150	2510	53.4	61.4
1—16	8.4	60	0.345	1.91	8.5	48	0.375	2.11	343	366	13.7	13.4	1600	1890	61.5	64.6
17—32	23.7	59	0.678	8.19	24.3	52	0.852	12.88	418	460	11.7	12.2	5080	6910	143	185
33—48	41.3	45	0.648	5.94	39.1	38	0.838	10.20	384	464	10.3	12.2	3530	5630	94.1	148
49—64	56.1	38	0.553	4.31	53.7	18	0.769	8.59	326	400	8.4	10.1	2520	4470	64.9	113
65—80	72.4	31	0.503	3.50	72.9	28	0.566	5.07	300	315	7.5	7.7	2130	2840	52.5	67.4

servation. The lower part of the figure shows the excretion in women and the upper part that in men. It should be noted that the results obtained in two men, 21 and 22 years old, are not included in the figure or in the calculations given below. This seems to be justified as the values obtained in these two cases were 1.54 and 1.38 mg./24 hrs. which deviate from all the others in a striking manner, and further more because it was not possible to obtain another 24-hour urine specimen as a control.

In each sex the material was divided up into various age-groups that is to say into groups comprising 5, 10 and 15 years of life. For each group the average age and average excretion were computed. In Table 2 some of these calculations are shown in detail. The average excretion of corticoids throughout life is illustrated graphically in Fig. 2 in which the upper curve represents the excretion in men and the lower one that in women. This graphic representation is drawn up on the basis of the mean values for each of the age-groups 0—9 years, 5—14 years, 10—19 years, and so on. A comparison of the results shown in Table 2 and Figs. 1and 2 clearly indicates a dependence of corticoid excretion on both age and on sex. During the first years of life the excretion is low (at an age of 2 about 0.20 mg /24 hrs) but it increases gradually year by year until a maximum is reached at the age of 25—30 years, when the mean excretion in women is about 0.69 mg./24 hrs and in men about 0 86 mg./24 hrs Throughout the following years only little change occurs and the mean excretion curves show a tendency to plateau formation. With advancing age, however, the excretion gradually decreases. Between 70 and 80 years of age the mean values amount to 0.49 and 0.55 mg /24 hrs. in women and men respectively.

The average excretion of corticoids in the two sexes behaves, on the whole, in a parallel manner but is on a higher level in men than in women (Fig 2) As is clearly seen from Fig. 1, a marked overlapping of the single values is found The sex difference of excretion is apparently less marked in children and in high age groups as compared with that in

in the case of women 98.6, 93.5 and 81.2 per cent respectively and in men 99, 93 and 77.2 per cent respectively.

It is hoped to use the present material as the basis for comparison when evaluating the corticoid excretion found in patients. For practical reasons which are to be discussed later on, it has been decided for this purpose to give rather wide limits for the normal excretion. The diagrams shown in Fig. 3 are made on the basis of these considerations. The average ex-

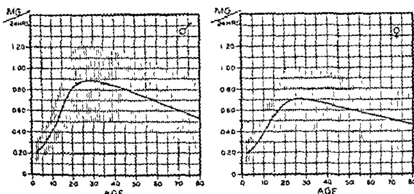


Fig. 3

Diagrams of the normal urinary excretion of corticoids Full-line curves· average excretion. Vertical hatched area area within which 100 per cent of the values for women, and 96.8 per cent of values for men are found Ordinate mg./24 hrs Abscissa Age.

cretion of corticoids in the two sexes is indicated by the full-line curves. The vertical hatched area shows the zone within which 100 per cent of the values range in women and 96.8 per cent of the values range in men.

Relation of the excretion of corticoids to body weight and to body surface area.

In order to examine if any relationship exists between the excretion of corticoids and body weight and surface area respectively a calculation was made on the basis of these factors for each individual subject The result of these calculations is

young adults. From the present material it is difficult to decide whether this apparent discrepancy really exists. In this connection it should be noted that relatively fewer subjects of high age were included in the study.

As appear from Fig. 1 a rather large individual variability

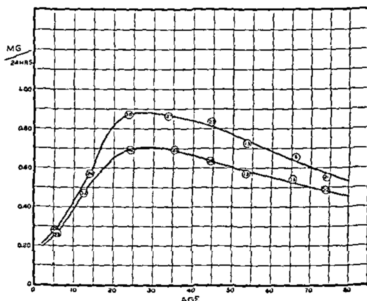


Fig 2.

The mean excretion of corticoids per 24-hours in men (upper curve) and in women (lower curve) The circles show the average excretion for 10-year age-groups (0—9 years, 10—19 years, etc). The figures in circles indicate the number of subjects in each age group. Ordinate Average excretion in mg/24 hrs. Abscissa: Age

of the values is found For a further examination of the magnitude of this variation the number of individual values deviating more than ± 50, 40 and 30 per cent from the values of the average curve were computed. In women these numbers were found to be 3, 14 and 40 respectively, and in men 2, 13 and 42 respectively. This means that the percentage number of individuals ranging *within* the above mentioned limits are

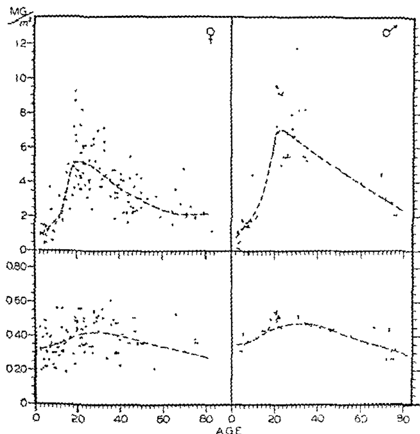


Fig 5.

The 24 hour excretion of corticoids (lower curves) and 17-ketosteroids (upper curves) expressed per square meter of body surface area
Ordinate mg. per sq m of body surface area Abscissa Age

curves for the absolute output values (Fig. 1) In adults up to the age of 50 the excretion per m² area is still a little higher than in children, whereas the opposite proportion is found in old age.

Paying no attention to age, the total material was then divided up into groups, partly according to body weight (10—19 kg, 20—29 kg and so on) and partly according to the area of the body surface (0.60—0.79 m², 0.80—0.90m² and so on) The average values for body weight, body surface and for the

shown in the two lower diagrams in Fig. 4 and 5 in which the individual values expressed as µg. per 24 hours and per kilogram of body weight, and as mg. per 24 hours per square meter of body surface area, respectively, are plotted against the chronological age. As appears from Fig. 4 the excretion per kg. of body weight is at the highest level in children (averaging

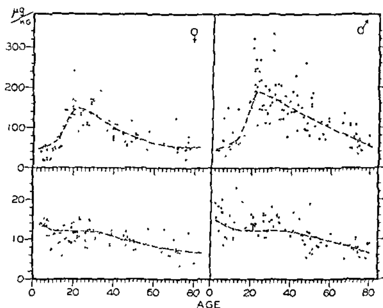


Fig 4.
The 24-hour excretion of corticoids (lower curves) and of 17-keto steroids (upper curves) expressed per kilogram of body weight Ordinate µg per kilogram of body weight and per 24 hours. Abscissa Age

14 5 µg per 24 hours at the age of 2—4). With advancing age the excretion decreases and between 70 and 80 years of age it ranges around 7 5 µg. per kg At an age between 10 and 40, however, the curves appear to have a tendency to plateau formation (Table 2). Furthermore, a considerable diminution of the difference in excretion between the sexes is seen. From Fig. 5 and Table 2 it appears that a calculating of the excretion per square meter of body surface area causes a levelling of the

found, but in this way too no direct ratio between excretion and weight was obtained.

The calculations carried out on the values for female subjects in a 20-year age-group (20—39 years) will be quoted here as an example. After dividing up this group into 4 weight groups: 45—53 kg., 54—60 kg., 61—67 kg. and 68—74 kg., the average body weights were found to be: 51.0, 57.2, 64.0 and 70.9 kg. respectively and the corresponding average excretion of corticoids was: 0.64, 0.67, 0.75 and 0.77 mg./24 hrs. (In order to avoid repetition the corresponding figures for the excretion of 17-ketosteroids will be mentioned here: 8.25, 7.81, 7.75 and 7.14 mg./24 hrs.).

Daily individual variations in the excretion of corticoids

When the determination of the corticoid excretion is carried out on a single 24-hour urine specimen only, the magnitude of variation in the excretion from day to day is among other factors of great importance in the evaluation of the result obtained.

For an examination of this problem determinations of the daily excretion were carried out on urine obtained from 3 women (A: 44 years, B: 17 years and K: 21 years) and 2 men (H: 46 years and R: 41 years). The examination of the women included daily analyses for a period of a month in order to see at the same time whether the menstrual cycle influences the corticoids in any way. The results are shown in Figs. 7 and 8 and in Table 3. The values for A and K fluctuate within the greater part of the area previously established for the normal excretion in women of their age-groups. No relation of excretion to the menstrual cycle is observed. In the case of woman B the variations were considerably less apart from two consecutive values of 0.78 mg. This increase has been difficult to explain as it is in all probability without any relation to the menstrual cycle which in this woman appeared to be irregular. (Menstruation ceased just before the assay period and set in again a week after the cessation of the assay. The greatest

total daily excretion of corticoids in each group were computed. The result of these calculations is charted in Fig. 6. A direct ratio of excretion to body weight is found only when the weight is under 60 kg. and 65—70 kg. in women and men respectively. Above these limits the excretion rises only a little with increasing weight and seems to approach a constant level. Similar relations are seen in the other part of the figure in

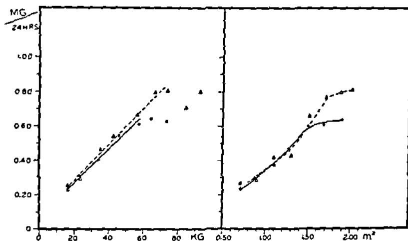


Fig 6.
The ratio of corticoids to body weight and to body surface area. ——— women. — — —. men. Ordinate: Excretion in mg. per 24-hours Abscissa. Kilogram of body weight (left part), sq m. of body surface (right part).

which the excretion is plotted against the body surface. The correlation between these two factors seems, however, to be not so good.

As the subjects in the high age-groups are to be found mainly within the weight groups above the mentioned limits, the absence of agreement here between excretion and weight could be due to the low absolute excretion of corticoids by these subjects.

For a further elucidation of this question calculations similar to those described above were carried out in some more narrower age-groups. On the whole, a better correlation was

found, but in this way too no direct ratio between excretion and weight was obtained.

The calculations carried out on the values for female subjects in a 20-year age-group (20—39 years) will be quoted here as an example. After dividing up this group into 4 weight groups: 45—53 kg., 54—60 kg, 61—67 kg. and 68—74 kg., the average body weights were found to be: 51.0, 57.2, 64.0 and 70.9 kg. respectively and the corresponding average excretion of corticoids was: 0.64, 0.67, 0.75 and 0.77 mg./24 hrs. (In order to avoid repetition the corresponding figures for the excretion of 17-ketosteroids will be mentioned here: 8.25, 7.81, 7.75 and 7.14 mg./24 hrs.).

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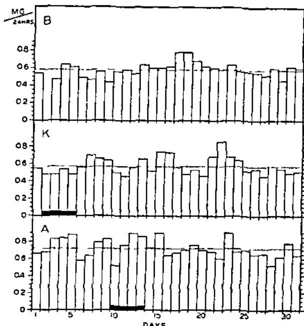


Fig 7.
Individual daily variations in the excretion of corticoids in 3 women (K and A· normal. B irregular menstruation). Black fields indicate the period of menses Ordinate· mg per 24-hours. Abscissa: days of experiment

Table 3
Daily variations of corticoid excretion in 3 men and 2 women as shown by the average values, the maximal and minimal excretion

$$S = \pm \sqrt{\frac{\Sigma d^2}{n-1}}$$

	Number of days	Corticoids mg/24 hrs. minimum	average	maximum	S
A ♀	31	0.52	0.72	0.90	0.11
B ♀	30	0.44	0.58	0.78	0.07
K ♀	31	0.46	0.57	0.85	0.10
H ♂	14	1.02	1.08	1.20	0.06
R ♂	7	0.90	1.09	1.20	0.11

variation for the 3 women was about ± 30 per cent of the mean for each woman (for K., however, a single value deviated + 49 per cent).

For the two men the daily variations were somewhat smaller, especially in H. as no value in this man deviated more than

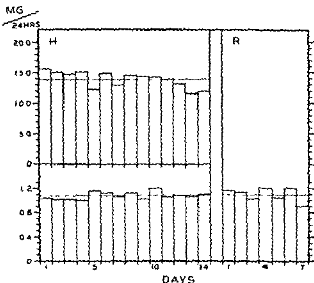


Fig 8.
Individual daily variations in the excretion of corticoids in 2 normal men (lower diagrams) and of 17-ketosteroids in case H (upper diagram). Ordinate mg per 24 hours. Abscissa days of experiment

10 per cent from his mean value For R. the corresponding figure was 17 per cent

In all the cases examined here the amounts of corticoids were entirely independent of the amount of urine excreted.

The urinary excretion of neutral 17-ketosteroids

The results of the 17-ketosteroid analyses carried out in this study confirm, on the whole, the results found by other investigators and therefore will only receive short comments

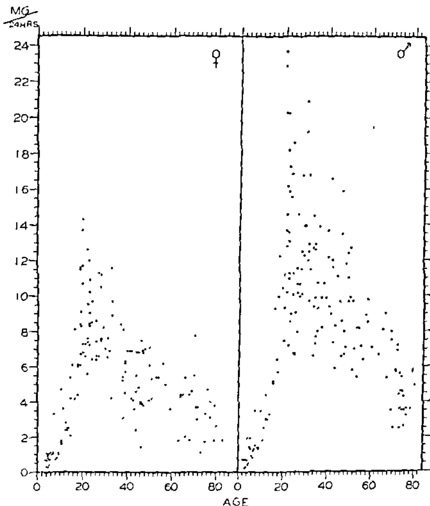


Fig 9.
The 24-hour urinary excretion of 17-ketosteroids in the subjects studied Each dot represents an individual value Ordinate mg per 24-hours Abscissa: Age

here The values obtained for the individuals are plotted in Fig. 9.

The excretion is very low during the early years of life and the increase is slow until the age of about 10—12 At this age an abrupt and steep increase commences and continues, in

both sexes, until a maximum is reached at an age between 20 and 25 years. From this point the excretion gradually decreases with advancing age.

Up to the age of 14—15 years the excretion is parallel and at the same level in both sexes, but after this it is considerably higher in men than in women. Thus the average outputs at the age of 24 years are 13 10 and 8.34 mg/24 hrs. in men and women respectively (see Table 2). Although less marked the sex difference in excretion is still distinct during the later decades.

The dispersion of the individual values in proportion to the mean was found to be somewhat greater than that found for the corticoids. This difference was most marked for the children under 10 years of age. After carrying out calculations similar to those previously described for the corticoids, it was found that for women over 10 years of age 15 of the individual values deviated more than 50 per cent and 28 more than 40 per cent from the values of the mean curve. In men of the same age the corresponding figures were 10 and 22. Hence the percentage of subjects with an excretion ranging within the stated limits and aged 10—80 years, must be 91 2 and 82.6 per cent in females and 93 5 and 85 7 per cent in males respectively.

All the individual values were also calculated on the basis of body weight and of body surface area. The results are shown in the upper diagrams in Figs 4 and 5 and in Table 2. The diagrams for the excretion expressed in this way have a pattern quite similar to that of the diagrams originally found for the absolute output values. In the narrower age-groups too no significant relation between the excretion and the weight or the surface was found. This is clearly indicated by the example mentioned on page 345.

The daily variations in excretion of 17-ketosteroids were studied in one case only (H. Fig. 8) During a period of 14 days the 24-hour excretion varied from 11.5 to 15 6 mg. The average excretion was 13 8 mg. and the dispersion ($S = \pm \sqrt{\frac{\Sigma d^2}{n-1}}$) 1.3. No value deviated more than 17 per cent from the mean

Comparison between the excretion of corticoids and of 17-ketosteroids.

As seen from the above a *clear difference in the excretion* of the two groups of steroids is found.

In view of the origin of the 17-ketosteroids the more striking sex difference in the excretion of these steroids is not unexpected.

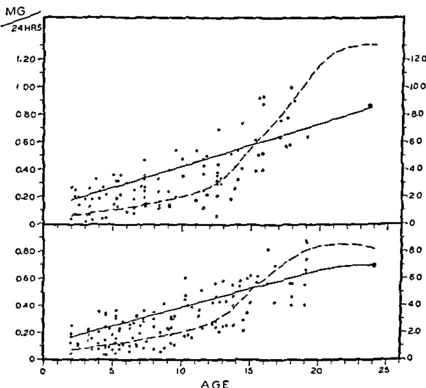


Fig. 10

Comparison of the excretion of corticoids with the excretion of 17-ketosteroids in normal children (lower diagram: girls, upper diagram boys).

● Individual values of corticoids

○. Individual values of 17-ketosteroids.

The full-line curves represent the mean excretion of corticoids and the broken curves that of 17-ketosteroids.

Ordinate: mg. per 24-hours (the left scale corticoids, the right scale. 17-ketosteroids) Abscissa Age

On the other hand, it is remarkable that the variations at different ages both in women and men are much more marked for the 17-ketosteroids than for the corticoids As appears from Fig. 10 this is true even during childhood. In children the excretion of corticoids increases in a linear manner with age as the increase year by year in boys amounts to about 0.030 mg. and in girls to 0 027 mg. per 24 hours Up to the age of 10—12 years the increase in excretion of 17-ketosteroids is less marked but at this stage an abrupt and a considerable change takes place as is indicated by the steep rise in the urinary excretion in both girls and boys. In both cases the curves for the average excretion intersect the corresponding mean curves of the corticoids at the age of about 15 years. This intersection is, of course, relative since the values for the 17-ketosteroids in this figure as well as in all the other ones shown here are charted on a scale 10 times smaller than are the values for the corticoids.

Fig. 11 illustrates the excretion of the two groups of steroids during all the age periods examined here. The full-line curves and the vertical hatched areas represent the excretion diagrams of the corticoids (see Fig. 3 and page 341). The excretion diagrams of the 17-ketosteroids are worked out accordingly to similar principles: the dot-and-dash-line represents the mean excretion and the dotted lines show the limits within which 99 per cent of the individual values for women and 97.1 per cent of those for men were found

It is obvious that only a slight alteration in the excretion of corticoids occurs between 20 and 40 years of age, whereas the 17-ketosteroid excretion exhibits a distinct maximum at the age just over 20 years after which the excretion immediately falls to lower values Thus the two mean curves intersect each other again, but whilst the first intersection took place exactly at the same age in the two sexes it now takes place at the age of 33 in women and at the age of 65 in men. In old age, at an age of 80, the average excretion both of corticoids and of 17-ketosteroids reaches values of a range similar to that found at the age of about 13—14 years.

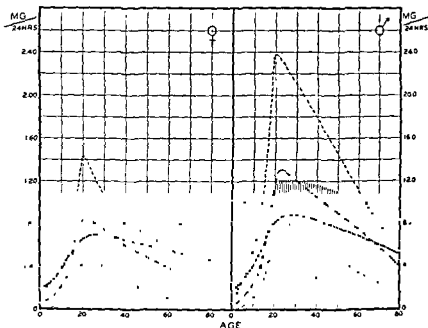


Fig 11

Comparison of the excretion of corticoids with that of 17-ketosteroids in all the normal subjects studied The full-line and the dot-and-dash line curves represent the average excretion of corticoids and 17-ketosteroids respectively The vertical hatched area, the area within which 100 per cent of the corticoid values for women and 96 8 per cent of the values for men are found. The broken curves: the limits within which 99 per cent of the 17-ketosteroid values for women and 97 1 per cent of the values for men are found Ordinate, mg. per 24-hours (left scale corticoids, right scale 17-ketosteroids) Abscissa: Age.

DISCUSSION

In the planning of this work two possibilities were contemplated. Firstly, to undertake a strict selection of a small number of subjects on the basis of a previous careful physical and laboratory examination of each individual and then to carry out the steroid analyses repeatedly and on different days. Secondly, essentially to base the selection of the normal persons subjects on their past history and then to carry out the

determination of the steroid excretion on a single 24-hour urine specimen from a very large number of subjects of both sexes and of all ages.

Consideration of the practical clinical use of the material for the evaluation of the corticoid excretion found in patients with various diseases has been the most important reason for the choice of the second plan. In patients the excretions is most often determined on a single 24-hour urine specimen and in order to evaluate the results [illegible]

[illegible]

When comparing the absolute values found in the present study with those reported in the literature, the different techniques used must be remembered. As was expected the values range between those given by *Heard, Sobel & Venning* (determination of reducing power of total neutral fraction) and those given by *Talbot, Albright et al* (determination of reducing power of water-soluble ketonic fraction). At the same time, however, it is interesting to note that the values are within the same range as found by other authors using the formaldehyde methods (see Table 1). The values found in children agree very closely with those reported by *King & Mason* (1950), who examined the excretion in a similar number of subjects of this age. For the adult subjects the results cannot be directly compared with those reported in the literature. Most of these reports only dealt with a small number of subjects and often without further information as to age

There is a reason to call attention to the close agreement with the results found by *Venning & Kazmin* (1946) using a biological determination of the excretion of glucocorticoids. By this method they observed similar variations in the excretion with regard to age and sex.

The variations in the excretion from day to day are of the same magnitude as found in several patients in whom daily analyses were carried out during periods of 4—14 days. In a similar manner *Talbot, Albright et al.* (1947) examined the daily excretion in 3 men and 1 women and found the chances

to be two out of three that the value for a single day would fall within ± 30 per cent of the average excretion for that individual.

King & Mason (1950) carried out single determinations of the daily excretion during 2—7 days in 6 children and found that the greatest variations amounted to approximately ± 30 per cent of the mean for each individual. *Heard* (1948) stated that the variations were about ± 50 per cent. Similar values were found by *Venning & Kazmin* (1946) when using biological determinations. The daily variations of 17-ketosteroid excretion (in case H.) correspond to the variation found by *Hamburger* (1948).

Some difficulties arise when giving an account of these day by day variations in excretion, as different factors can play a part. An account of the technical variation of the analysis was given previously (*Sprechler* 1950 a). This error may be responsible for only a small part of the variation. Inaccuracies in the urine collection need not to be taken into account in the cases examined here. Nevertheless, the decisive importance of a perfectly accurate 24-hour demarcation of the urine collection must be pointed out here This is very important, especially in cases in which the analysis is carried out on a single 24-hour specimen only. In children the collection must be completed under special supervision In subjects of great age one must be aware of incontinence of urine and especially of the presence of retention of urine in the bladder which can account for a rather large error, especially in cases in which there is at the same time a low 24-hour excretion of urine.

The remaining possibility is the spontaneous daily variation in excretion. In several cases it was found that the amounts excreted were independent of the volume urine excreted, and it is obvious to assume, therefore, that the variations actually reflect a varying activity of the adrenal cortex This assumption agrees very closely with our present conception of hypophysis-adrenal cortex (probably also involving the adrenal medulla and hypothalamus) as an accurately correlated system. The functional state of this system depends

closely on the demands made on the organism. An increase of these demands which, under normal conditions, is often due to increased physical (and in part psychical) output of work, causes via the hypophysis a stimulation of the adrenal cortex resulting in an increased production and secretion of active adrenal cortical steroids. A decrease of the demands, as for instance during sleep or other forms of reduced activity, results in a decreased function. This is well illustrated by the previously mentioned investigations on the diurnal variations in excretion of both corticoids and of 17-ketosteroids (*Pincus, Romanoff & Carlo*, 1948). The high urinary excretion of corticoids could be explained as the effect of stress due to the abrupt change of activity in relation to wakening (*Selye*, 1947).

The present investigations were performed under conditions of an unaltered mode of life for the subjects examined. Bearing the above in mind, some of the variations found from day to day can be explained. From other sources it is shown, that one can expect to find a lower excretion in subjects confined to bed for some time, and one cannot exclude that smaller daily variations in excretion should also be found here

By the use of the diagrams (Fig. 3) on the normal excretion of corticoids for the evaluation of the excretion found in patients, it is necessary to pay due regard to the factors described above. On the basis of an analysis carried out on a single 24-hour specimen one must be cautious to record »a high normal« or »a low normal« excretion Such designations are, however, justified when the average content of several 24-hour specimens is determined, or, for example, if all of the individual values for a large number of patients within the same group of disease are all high or all low.

The changes associated with the menstrual cycle do not apparently influence the adrenal cortical function as reflected by the urinary excretion of corticoids This finding is in agreement with the results found by *Venning & Kazmin* (1946) using a biological method for the determination of the urinary excretion of glucocorticoids in two women during the cycle. Contrary to these findings *Davis & Hulit* (1949) observed a

to be two out of three that the value for a single day would fall within ± 30 per cent of the average excretion for that individual.

King & Mason (1950) carried out single determinations of the daily excretion during 2—7 days in 6 children and found that the greatest variations amounted to approximately ± 30 per cent of the mean for each individual. *Heard* (1948) stated that the variations were about ± 50 per cent. Similar values were found by *Venning & Kazmin* (1946) when using biological determinations. The daily variations of 17-ketosteroid excretion (in case II.) correspond to the variation found by *Hamburger* (1948).

Some difficulties arise when giving an account of these day by day variations in excretion, as different factors can play a part. An account of the technical variation of the analysis was given previously (*Sprechler* 1950 a). This error may be responsible for only a small part of the variation. Inaccuracies in the urine collection need not to be taken into account in the cases examined here. Nevertheless, the decisive importance of a perfectly accurate 24-hour demarcation of the urine collection must be pointed out here. This is very important, especially in cases in which the analysis is carried out on a single 24-hour specimen only. In children the collection must be completed under special supervision. In subjects of great age one must be aware of incontinence of urine and especially of the presence of retention of urine in the bladder which can account for a rather large error, especially in cases in which there is at the same time a low 24-hour excretion of urine.

The remaining possibility is the spontaneous daily variation in excretion. In several cases it was found that the amounts excreted were independent of the volume urine excreted, and it is obvious to assume, therefore, that the variations actually reflect a varying activity of the adrenal cortex This assumption agrees very closely with our present conception of hypophysis-adrenal cortex (probably also involving the adrenal medulla and hypothalamus) as an accurately correlated system. The functional state of this system depends

ther details concerning age, sex and the absolute values were given.

During the last two years our knowledge concerning the excretion of 17-ketosteroids in normal human subjects, has on the whole, been clarified. Several extensive investigations on this subject have been carried out at different places. In the present study, therefore, the results obtained for the excretion of these steroids in the subjects examined may serve as a control for the normal adrenal cortical function involved in the excretion of 17-ketosteroids. In men this is, of course, only partly valid since a fraction of these steroids originates in the testis.

Fig. 12 illustrates the diagrams of 17-ketosteroid excretion in men and women as previously mentioned (Fig 11). The dotted curves and the dot-and-dash ones indicate the mean excretion as given in the investigations referred to Apart from slightly lower values at the age of between 20 and 30 years a close agreement with the mean curves reported by *Hamburger* (1948) is found. In men a similar curve has been found by *Robinson* (1948). The mean values reported by *Kirk* (1949) and by *Miller & Mason* (1945) are identical with those found in the present investigation. The last mentioned group of authors examined the excretion in a large number of children at the age of between 11 and 18 years Using another technique *Kenigsberg, Pearson & McGavack* (1949) obtained somewhat higher values whereas the mean values reported by *Hamilton & Hamilton* (1948) are slightly lower. The disagreement between the absolute values obtained by the last mentioned authors and those found by the above mentioned authors seems to be due to technical differences of methods as a parallel course of the mean curves is observed in all cases

The conclusion arrived at by this comparison must be that with regard to the excretion of 17-ketosteroids, the present material does not differ essentially from the materials which have been used previously by other investigators for the determination of the normal excretion of 17-ketosteroids.

More interesting problems arise on comparing the differences

fall in the number of circulating eosinophils at the time of ovulation and claimed it to be an expression of an increased adrenal cortical function. In all probability, however, this reaction must be judged with great caution. It is remarkable too, that the same authors did not find any convincing alteration in the number of circulating eosinophils during pregnancy when the adrenal cortical function is known to be considerably increased (*Venning*, 1946).

In 1948 it was stated by *Day* that the excretion of corticoids as expressed per kilogram of body weight was considerably higher in the newborn than in adults. *Matson & Longwell* (1949) confirmed this by an examination of 19 newborn boys and a few adults. The excretion per kg. of body weight was found to be 6 times higher in the newborn than in adults.

The calculations carried out in the present study do not include children of an age under 2 years, but on the other hand there are a considerably larger number of individuals of the other age-groups. The mean excretion at the age of 2 is found to be higher than that in adults. The values for the corticoid excretion in newborn children reported in the literature correspond, however, rather closely to the values obtained by an imaginary lengthening of the mean curves for the corticoids in Fig. 10. At the age of zero the mean excretion would be found to range from about 0.10 to 0.11 mg. per 24 hours. Assuming the birth-weight to average 3.3 kg., the corticoid excretion would be approximately 30—33 μg pr kilo. of body weight. When carrying out similar calculations on the values of the glucocorticoid excretion reported by Venning and her associates corresponding results are found.

As mentioned in the introduction, *Talbot* (1949) recently claimed that the amount of corticoids excreted per square meter of body surface area is of the same magnitude during the whole of life. The results (Fig. 5) obtained in the present study are to some extent in disagreement with this opinion. Comparisons with the work referred to cannot be made directly as only a small number of adults were examined and no fur-

relationship and, furthermore, that the curves of boys and of girls were identical. Up to the age of 15 a similar relationship between 17-ketosteroid and age is found in the present study.

In boys the abrupt increase in excretion of neutral 17-ketosteroids might be explained by an incipient production of androgens in the testis. As a uniform excretion is found in boys and in girls it would mean, however, that part of the 17-ketosteroids in girls must originate in the ovaries. There is, however, much evidence against such a conception. Thus in most of the investigations carried out on ovariectomized women a normal excretion of 17-ketosteroids is found and moreover, no marked fall in the excretion is observed at the menopause. An oestrogenic stimulation of the adrenal cortex produced via the hypophysis cannot be entirely excluded as it is known that oestrone administered to animals may produce a hypertrophy of the adrenal cortex provided that the hypophysis is intact (*Christensen*, 1944). Lastly an alteration in the intermediate steroid metabolism after the age of 10 resulting in a greater conversion of corticoids into 17-ketosteroids after this age might be considered as a possible explanation It is difficult, however, to accept this conception as it is known that various steroids administered to children are transformed and excreted in the same way as in adults (*Talbot & Sobel*, 1947).

It is quite reasonable to assume that the precursors of neutral 17-ketosteroids are elaborated in the adrenal cortex in girls as well as in boys. As it has been shown that corticoids and 17-ketosteroids are excreted independently of each other it is reasonable to assume that they also originate independently of each other as a result of two functional activities of the adrenal cortex. While the ability to elaborate corticoids is fully developed from birth, the other function does not assert itself until the age of 10—12 years It is difficult to decide whether the ability of the adrenal cortex to elaborate precursors of 17-ketosteroids (including androgenic substances) is developed slowly during the first few years of life or whether it starts only at the above mentioned age. It is possible that

observed between the excretion of corticoids and of 17-ketosteroids. In children the amount of urinary corticoids increases gradually and uniformly with advancing age showing a straight line curve. Up to the age of 10—12 years, on the contrary, only small amounts of 17-ketosteroids are present in

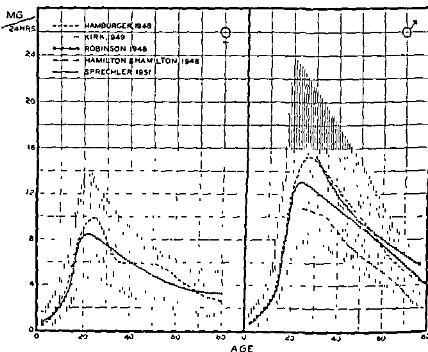


Fig 12
Normal excretion of 17-ketosteroids Full line curves the average excretion Vertical hatched area the area comprising 99 per cent of the values for women and 97 1 per cent of the values for men For comparison the mean curves of normal excretion found by other authors are shown Ordinate mg per 24-hours Abscissa Age

the urine and the increase in excretion is only slight. At this point an abrupt change takes place, indicated by a steep increase in the excretion of 17-ketosteroids during the subsequent years. The excretion of androgenic hormones behaves in a similar manner and by plotting the logarithm of the amounts excreted against age *Dorfman* (1948) found a linear

maxima observed in both sexes are due to steroids derived from the adrenal cortex, since, as stated by *Hamburger, Halvorsen & Pedersen* (1945) the maximal excretion of androgenic hormones in men (i. e. chiefly derived from the testis) is reached only at a later age (30—40 years).

In order to examine more closely whether the established mean curves of steroid excretion offer an actual measure of the functional state of adrenal cortex at the various ages, it is necessary to pay attention to a few details concerning the mode of action of the adrenal cortical steroids Our knowledge of this subject is still rather scanty as it is essentially limited to a recording of a number of secondary phenomena, and hence a detailed discussion would be outside the scope of this work.

The corticoids are of decisive importance for the continuance of life so that they must be produced during the whole of life. The actual nature of the mode of action is unknown but a large amount of evidence suggests that they occupy important links in certain intermediate metabolic processes going on in the cells of the organism. Increased activity of cells produces (via the hypophysis) an increased elaboration and secretion of corticoids from the adrenal cortex (*Sayers & Sayers*, 1948) This also means that the need, and consequently the production (secretion), of corticoids depends on the total amount of body protoplasm.

Provided the conception of the corticoids broadly outlined here is correct, one might expect a certain relationship between the corticoid excretion and the metabolic rate of the organism. From our knowledge of the total metabolic rate in the organism it would be expected that the total production of corticoids is: 1) lowest in children (small amount of protoplasm, lowest total metabolic rate), 2) largest in adults, but higher in the younger subjects than in the older ones (decreasing metabolic activity of cells in old age), 3) larger in men than in women (larger amount of protoplasm and higher metabolic rate in the former). Furthermore, it is reasonably to expect that the production of corticoids as expressed per kilogram of body weight or per square meter of body surface area will be

the small amounts of 17-ketosteroids excreted in the urine before that time originates from a conversion of corticoids into 17-ketosteroids. This possibility is supported by the fact, that a rupture of the side chain at C-17 in the molecule of corticoids resulting a 17-ketosteroids can take place in the process of steroid metabolism in the organism. An increase of urinary 17-ketosteroids may be seen, for example, during the administration of Cortisone to patients.

The explanation outlined above on the observed excretion pattern of the two groups of steroids is further supported by the data obtained in a previous investigation on the excretion of steroids during ACTH administration to children and to adults (*Sprechler*, 1950 b). The useal response in adults was indicated by a marked increase in the excretion of both corticoids and 17-ketosteroids. In children a similar response was *seen in the case of corticoids whereas 17-ketosteroids were* influenced only slightly or not at all. After ACTH administration to a 10 year old girl for some time an alteration in the excretion rate occurred approaching the conditions found in adults. An acceleration of the above mentioned »action of maturation« of androgenic function of the adrenal cortex (or the ability to elaborate the precursors of 17-ketosteroids) due to the continued unphysiological high stimulation might explain this observation The occurrence of such alterations in the adrenal cortex is known from human pathology and it is not unlikely that some cases of precocious puberty may be connected with such an overstimulation of the adrenal cortex.

On examining the further course of the excretion curves, a distinct maximum of the 17-ketosteroids is observed immediately after the twentieth year. A little later the corticoids reach maximal values of excretion but in the case of these steroids the excretion continues essentially at the same level throughout the following 20 years. After the age of 15 some difficulties arise when comparing the excretion of 17-ketosteroids in men with that in women, as the development of the androgenic function of the testis in the former accounts for a considerably larger excretion. It is apparent, however, that the pronounced

between the metabolic rate and the production and excretion of corticoids presuppose that the chemical tests actually measure the glucocorticoids and their metabolites. During recent years it has been customary to divide the corticoids into two groups: The *glucocorticoids* and the *mineralocorticoids* (or: 11-oxy- and 11-desoxy-corticoids respectively). Both groups of corticoids are included in the chemical assay by the method used in the present study (as is also the case with the technique commonly used in the formaldehyde methods). Comparisons of the results obtained by a simultaneous biological and chemical determination of the corticoid excretion in urine have, however, shown a marked agreement between the reducing power and the glucocorticoid activity (*Sprechler*, 1950, a, b). Furthermore, recent studies of *Weissbecker & Staudinger* (1951) *seem to throw a good deal of doubt on the significance of the so-called mineralocorticoids in urine.* They were able to separate the two groups of *steroids* and afterwards to obtain a separate determination of each individual group. By this means it was shown that the »11-desoxy-corticoids« form only a small part of the total amount of corticoids in the urine and that the excretion does not increase during ACTH administration. Furthermore, no alteration in the excretion was found in various diseases (i. a. chronic rheumatic diseases) in which a decreased excretion of the »11-oxy-corticoids« was nevertheless observed.

For the 17-ketosteroids, the arguments discussed above do not hold. The activity of their precursors (in women presumably *elaborated exclusively in the adrenal cortex) are unknown and it is quite impossible to form any idea as to the amount actually produced.*

SUMMARY

The aim of the present work was: 1) to examine the normal urinary excretion of corticoids in men and in women of all ages and to establish the limits of the normal range. 2) to examine the daily individual variations in excretion (examin-

maximal in children followed by a decrease with advancing age.

These statements agree remarkably well with the observations made on the excretion of corticoids. Provided the above theory is correct, the corticoids seem to offer a reasonably good measurement of the adrenal cortical activity throughout life.

It should be pointed out that one cannot expect any agreement between the values of corticoids found in the present material and those results which would have been obtained by carrying out determinations of the *basal* metabolic rate of the individuals. This is clearly illustrated, for example, by examining the excretion expressed per square meter of body surface. In order to examine whether such a close relationship exists it would be necessary to carry out all these investigations under standard conditions paying due attention to diet, temperature, etc. and keeping the subject completely at rest during the whole period of urine collection. When calculating the results obtained in this way, factors similar to those used for the calculation of the standard metabolic rate must be considered.

The mean excretion of corticoids was found to be greater in men than in women This difference is considerably decreased, but not altogether abolished when the excretion is expressed per kilogram of body weight. There is a possibility that small amounts of androgenic hormones produced in the testis are converted by the steroid metabolism in the organism into steroids, which are involved in the assay procedures and interfere with the final chemical determination. Against this view, however, are the facts that the difference was also found in children, and that no rise in urinary corticoids resulted from the administration of testosterone to patients For these reasons the difference observed must actually be the expression of a higher adrenal cortical function in men than in women. This finding moreover, can be brought within the scope of the above mentioned theory.

All the above mentioned considerations on the relationship

When expressing the excretion per kilogram of body weight the values are largest in children (at the age of 2 ranging around 14.5 μg. per kilogram) and lowest in the high age groups (at the age between 70 and 80 years ranging around 7.5 μg. per kilogram). At the age of between 10 and 40 years only a small change in the values occurs. When calculating the corticoid excretion in this way, a considerably diminution of the sex differences in excretion is observed. A certain relation between corticoid excretion and body weight seems to exist within narrower age-groups.

The variations in excretion with age are considerably diminished when calculating the values per square meter of body surface area. Expressed in this way, the excretion in adults up to an age of 50 is, however, still a little higher than in children, whereas after that age the values are lower than in children.

The greatest daily variations in excretion in the 3 women examined throughout a month were approximately ± 30 per cent of the mean value for each individual. The variations day by day in the 2 men examined for 14 and 7 days respectively were somewhat smaller. In women no relation of excretion to menstrual cycle was observed.

The values found for the excretion of 17-ketosteroids did not differ from those reported in the literature. It is pointed out that appreciable amounts of these steroids are excreted only after the age of 10—12, and that the variations with age and sex respectively are much more marked than for the corticoids. The dispersion of the individual values is very great in children, and in adults somewhat greater than for the corticoids. The excretion of 17-ketosteroids seems to be without any relation to body weight and body surface area respectively.

The investigations definitely show that corticoids and 17-ketosteroids are excreted independently of each other. The conclusion is drawn that the two groups of steroids originate in the adrenal cortex from two separate functions which are developed at a different period of life.

ing at the same time if any relation exists between excretion and the menstrual cycle in women) and 3) to study the relation between the excretion of corticoids and that of 17-ketosteroids.

An account of the investigations reported in the literature on the normal excretion of corticoids is given. At present it is only in the case of children that there are available a few careful investigations on a large number of subjects. It is pointed out that a direct comparison of the results in more cases cannot be made as various techniques have been used for the analyses.

The relation of the excretion of 17-ketosteroids to age and sex has been established during recent years. The most important reports concerned with this subject are briefly discussed.

Our *own investigations* consist of determinations of the urinary 24-hour excretion of corticoids in 214 women of an age from 2 to 83 years and in 187 men of an age from 2 to 80 years At the same time determinations of 17-ketosteroid excretion were carried out In 3 women the corticoid excretion was assayed daily throughout a period of a month, and in 2 men daily for 14 days and 7 days respectively.

The corticoids were determined chemically by the method previously described, which is based upon the reducing properties of these steroids (the determination is made on the neutral ketonic steroids extracted from urine at pH 1 by means of chloroform). The 17-ketosteroids were assayed by the micromethod devised by *Hamburger*

The results on corticoids indicate a daily excretion approximately parallel in the two sexes during life but a little higher in men than in women. The excretion is smallest in children, largest in adults of the age between 20 and 40 (being on an average about 0 69 mg. in women and about 0.86 mg. in men per 24 hours), and falling again in subjects of high age-groups. A rather large variation of the individual excretion is found, but only in a few cases (3 women and 2 men) did the values deviate more than ± 50 per cent from the values of the mean curves.

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The excretion (and production) of corticoids seems to be related to the activity of the cells (and to the metabolic rate of the body). Presumably the corticoids are of decisive importance in certain intermediate metabolic processes, though the actual mechanism is as yet unknown. These problems are discussed in detail.

ACKNOWLEDGEMENT

I am greatly indebted to the National Danish Association against Rheumatic Diseases for grants in aid of the present work.

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SUMMARY IN DANISH

I

En oversigt over corticosteroiderne særlig med henblik paa udskillelsen i urinen hos normale og i patologiske tilfælde

Der gives en kort omtale af de vigtigste historiske data vedrørende binyrebarkens betydning, binyrebarkekstrakternes fremstilling og de enkelte steroiders isolering og syntese.

Efter en redegørelse for corticosteroidernes kemiske struktur fremhæves det, at bestemte træk i molekylstrukturen er af afgørende betydning for den biologiske aktivitet. Paa basis af denne er det almindeligt at inddele corticosteroiderne i 2 hovedgrupper glucocorticoider og mineralocorticoider Men desuden dannes i binyrebarken steroider med sexualhormonlignende egenskaber.

De talrige biologiske metoder, der er angivet til paavisning af corticoiderne, gennemgaas skematisk. Det fastslaas, at kun to af dem er tilstrækkelig følsomme til en kvantitativ bestemmelse af de smaa mængder aktive corticoider, der udskilles i urinen, nemlig den saakaldte »kulde-test« og »leverglycogenaflejrings-testen«. Kun sidstnævnte har fundet praktisk anvendelse

De kemiske metoder, der siden 1945 har været foreslaaet til kvantitativ bestemmelse af corticoiderne i urinen, beskrives ret detailleret, og den praktiske anvendelse af dem diskuteres.

Endelig gives, i delvis skematisk form, en fremstilling af de forholdsvis faa resultater, der i litteraturen er meddelt om ud-

III

Undersøgelser over udskillelsen af corticoider og 17-ketosteroider i urinen under tilførsel af adreno-corticotropt hormon (ACTH).

Hovedformaalet har været at undersøge, om der ved en bestemmelse af udskillelsen af corticoider og 17-ketosteroider i urinen kan opnaas et kvantitativt maal for virkningen af ACTH hos børn og voksne Desuden har det været hensigten at undersøge, om særlige faktorer eventuelt kan indvirke paa binyrebarkens reaktion overfor ACTH-stimulationen.

Undersøgelserne omfatter bestemmelser af udskillelsen af corticoider og af 17-ketosteroider i urinen før, under og efter tilførsel af ACTH til 25 patienter (2 børn, 15 kvinder og 8 mænd) lidende af forskellige sygdomme.

1) a. Karakteristisk for de normale udskillelseskurver for steroiderne under ACTH-tilførsel viser sig at være en stigning i udskillelsen allerede den første dag, og denne fortsætter progressivt indtil et maksimum naas 3—5. dag. Den maksimale binyrebarkreaktion maalt ved steroidudskillelsen er afhængig af doseringen, men samtidig udsat for temmelig store individuelle variationer.

b I 4 tilfælde er fundet en nedsat binyrebarkfunktion Resultaterne af steroidanalyserne sammenlignes med de resultater, der opnaaedes i tilslutning til en 48 timers ACTH-funktionsprøve, der blev udført paa en kvindelig patient med mb Addison. Steroidudskillelsen stiger uregelmæssigt og langsomt, og der kommer intet tydeligt maksimum. Efter seponering af ACTH vedvarer den træge reaktion, idet udskillelsen først efter flere dages forløb naar det plan, der fandtes før ACTH indgiften begyndte. I nogle tilfælde var dette særlig fremtrædende for corticoidernes vedkommende, hvorfor man ved udførelsen af den af Thorn angivne 48 timers ACTH-test maa overveje ogsaa at foretage disse analyser.

2) I 2 tilfælde, hvor ACTH blev givet i varierende doser, beregnedes gennemsnitsudskillelsen af corticoider og 17-ketosteroider svarende til hvert dosisplan. Naar de herved op-

skillelsen af corticoider hos normalpersoner og hos patienter med forskellige lidelser i hypofyse og binyrebark visende sig ved hypo- eller hyperfunktion af disse.

II

Undersøgelser over den kemiske bestemmelse af corticoider i urinen.

Formaalet har været at indarbejde en kemisk metode til kvantitativ bestemmelse af corticoidudskillelsen i urinen. Trods en grundig experimentel undersøgelse af metoderne angivet af *Heard & Sobel* og af *Talbot, Saltzman, Wixom & Wolfe* lykkedes det ikke at opnaa tilfredsstillende resultater med nogen af dem. I førstnævnte hurtige og lette metode laa vanskeligheden i at undgaa uspecifike stoffer, da reduktionsbestemmelsen udføres direkte paa det totale urinekstrakt, medens det i sidstnævnte langvarige og besværlige metode var umuligt at opnaa paalidelige kolorimetriske bestemmelser af reduktionen.

Ved at kombinere principperne i disse to metoder er det lykkedes at komme udenom de nævnte vanskeligheder.

For at fastlægge de optimale betingelser er der foretaget en experimentel undersøgelse af de forskellige trin i analysen, der i princippet gaar ud paa at bestemme reduktionsevnen af de lipoid-opløselige ketoniske corticoider, der udrystes af urin ved pH 1.

Relationen mellem de reducerende corticoider og de biologisk aktive glucocorticoider er undersøgt ved samtidige kemiske og biologiske analyser af urin fra patienter, der er behandlet med ACTH. De stigninger og fald i udskillelsen, der herved er fundet, forløber parallelt for de to metoder, hvilket tyder paa, at de afspejler binyrebarkens funktion paa en entydig maade. Udskillelsen af de biologisk aktive corticoider synes i tilslutning til stimulationen at stige procentisk mere end de reducerende corticoider. De eventuelle aarsager hertil diskuteres.

Noget lignende er iagttaget hos et barn, men her kun med hensyn til corticoidudskillelsen. Der diskuteres muligheden af antistofdannelse.

Til slut omtales kort de steroidanalyser, man kan tænke sig at udføre for at bedømme binyrebarkens reaktion i kliniske forsøg med ACTH.

IV

Undersøgelser over den biologiske bestemmelse af glucocorticoider.

Formaalet har været at indarbejde en af de foreliggende biologiske metoder til glucocorticoidbestemmelse.

Efter 12—24 timers faste tømmes adrenalektomerede dyrs lever for glykogen. Glucocorticoiderne er imidlertid i stand til at gendanne leverglykogenet, og baseret herpaa har *Venning, Kazmin & Bell* (1946) udarbejdet en metode til kvantitativ bestemmelse af disse steroider i urinen. Denne metode har været undersøgt her og er fundet anvendelig til det nævnte formaal.

Det er vigtigt stadig at kontrollere musenes følsomhed overfor den glucosedosis, der gives samtidig med steroiderne, da denne i sig selv kan bevirke en vis glykogenaflejring

Forsøg med urinekstrakter, der er opnaaet fra patienter i perioderne før og under indgift af ACTH, synes at tyde paa, at der under stimulationen kommer en kvalitativ ændring i sammensætningen af corticoiderne i urinen, idet dosis-virknings kurven faar et stejlere forløb Dette kan forklare nogle af de uoverensstemmelser, der under ACTH indgift tidligere er fundet mellem den procentiske stigning af de kemiske og de biologiske værdier for corticoidudskillelsen. Som standard har været anvendt Cortison. Dette er som følge af ovenstaaende fund muligvis ikke berettiget, hvis der foreligger en hyperfunktion af binyrebarken.

Meget lave værdier for udskillelsen er fundet i 7 tilfælde af Addison's sygdom og i et tilfælde med nedsat hypofysefunktion.

naaede værdier for udskillelsen afbildes i et koordinatsystem overfor logaritmen til dosis findes en linær relation Disse kurver sammenholdt med resultaterne af de øvrige undersøgelser viser, at der for at fremkalde en stigning i corticoidudskillelsen hos børn mindst skal tilføres 5—6 mg. ACTH og hos voksne ca. 12 mg. pr. døgn, medens de tilsvarende doser for 17-ketosteroidernes vedkommende er henholdsvis ca. 12 og ca. 14 mg. pr. døgn. Visse forhold taler for, at de nævnte doser svarer til de mængder ACTH, der normalt produceres i menneskets hypofyse.

3) I 3 tilfælde, hvor ACTH er givet i høje doser, er der 9.—10. dag observeret et midlertidigt fald i corticoidudskillelsen; i et af tilfældene gælder dette ogsaa 17-ketosteroidudskillelsen En udmattelse af binyrebarken som en mulig forklaring herpaa diskuteres. Det fremhæves, at det maaske kan være uheldigt i hver enkelt injektion at tilføre den mængde, binyrebarken maksimalt kan udnytte. Denne mængde synes at ligge mellem 20 og 30 mg.

4) I de fleste tilfælde er der i tilslutning til indgift af ACTH observeret en procentisk større stigning i udskillelsen af corticoider end af 17-ketosteroider, hvilket i reglen har været fremtrædende allerede fra den første dag. Denne forskel i udskillelsesforholdene er særlig tydelig hos børn, hvor der i 2 tilfælde kom en ligesaa kraftig stigning i corticoidudskillelsen, som man ser hos voksne, medens 17-ketosteroidudskillelsen kun steg lidt I det ene tilfælde iagttoges efter 50 dages ACTH indgift en ændring i binyrebarkreaktionen, der tages som et udtryk for en begyndende modning af binyrebarkens »androgene« funktion Undersøgelserne tyder paa, at corticoiderne og 17-ketosteroiderne produceres uafhængigt af hinanden i binyrebarken, men at dennes evne til at danne 17-ketosteroidernes forstadier først udvikles paa et sent tidspunkt (formentlig samtidig med konsmodningen)

5) I langtidsforsøg er der hos 2 voksne efter henholdsvis 70 og 40 dages ACTH indgift iagttaget en refraktær tilstand, hvor steroidudskillelsen naar værdier, der fandtes for forsøgets begyndelse, og hvor den ikke stiger selv efter højere doser.

Noget lignende er iagttaget hos et barn, men her kun med hensyn til corticoidudskillelsen. Der diskuteres muligheden af antistofdannelse.

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Efter 12—24 timers faste tømmes adrenalektomerede dyrs lever for glykogen. Glucocorticoiderne er imidlertid i stand til at gendanne leverglykogenet, og baseret herpaa har *Venning, Kazmin & Bell* (1946) udarbejdet en metode til kvantitativ bestemmelse af disse steroider i urinen. Denne metode har været undersøgt her og er fundet anvendelig til det nævnte formaal.

Det er vigtigt stadig at kontrollere musenes følsomhed overfor den glucosedosis, der gives samtidig med steroiderne, da denne i sig selv kan bevirke en vis glykogenaflejring.

Forsøg med urinekstrakter, der er opnaaet fra patienter i perioderne før og under indgift af ACTH, synes at tyde paa, at der under stimulationen kommer en kvalitativ ændring i sammensætningen af corticoiderne i urinen, idet dosis-virknings kurven faar et stejlere forløb. Dette kan forklare nogle af de uoverensstemmelser, der under ACTH indgift tidligere er fundet mellem den procentiske stigning af de kemiske og de biologiske værdier for corticoidudskillelsen. Som standard har været anvendt Cortison. Dette er som følge af ovenstaaende fund muligvis ikke berettiget, hvis der foreligger en hyperfunktion af binyrebarken.

Meget lave værdier for udskillelsen er fundet i 7 tilfælde af Addison's sygdom og i et tilfælde med nedsat hypofysefunktion.

Udskillelsen af glucocorticoider blev bestemt hos 2 normale kvinder og 14 normale mænd. Gennemsnitsudskillelsen var henholdsvis 35 µg. og 64 µg. pr. døgn. En række 48 timers urinopsamlinger fra en kvinde (med kronisk polyarthritis) undersøgtes i en periode paa 22 dage. Udskillelsen varierede mellem 25 og 89 µg. pr. døgn med en gennemsnitsværdi paa 48 µg.

V

Undersøgelser over den normale udskillelse af reducerende corticoider i urinen hos mennesket og corticoidernes relation til 17-ketosteroiderne.

Formaalet med dette arbejde har været 1) at undersøge den normale udskillelse af corticoider i urinen hos mænd og kvinder i alle aldre og at fastlægge grænserne for denne, 2) at undersøge de daglige variationer i udskillelsen (herunder ogsaa, om udskillelsen har nogen relation til menstruationscyklus), samt 3) at undersøge relationen mellem corticoidudskillelsen og 17-ketosteroidudskillelsen.

Der redegøres for de i litteraturen meddelte undersøgelser over den normale corticoidudskillelse. Kun for børns vedkommende foreligger enkelte grundige undersøgelser over dette emne Det pointeres, at den anvendte teknik ved analyserne har været noget varierende, hvorfor en direkte sammenligning i flere tilfælde ikke lader sig gøre.

Forholdene vedrørende den normale udskillelse af 17-ketosteroider er klarlagt i løbet af de sidste aar. De store arbejder, der foreligger herom, omtales kort

Egne undersøgelser omfatter bestemmelser af døgnudskillelsen af corticoider hos 214 kvinder i alderen 2—83 aar og 187 mænd i alderen 2—80 aar Der er samtidig foretaget bestemmelser af 17-ketosteroidudskillelsen Hos 3 kvinder er corticoidudskillelsen bestemt dagligt i 1 maaned, hos 2 mænd dagligt i henholdsvis 14 dage og 7 dage

Corticoiderne er bestemt med den tidligere beskrevne metode, der er baseret paa disse steroiders reducerende egenska-

her (reduktionsbestemmelsen foretages paa de neutrale ketoniske steroider, der med chloroform lader sig ekstrahere af urin ved pH 1). 17-ketosteroiderne er bestemt rutinemæssigt med den af *Hamburger & Rasch* beskrevne mikrometode.

Resultaterne viser, at corticoidudskillelsen forløber nogenlunde parallelt hos de to køn, men er lidt større hos mænd end *hos kvinder. Udskillelsen er mindst hos børn, størst i 20—40* aars alderen (gennemsnitlig her for kvinder ca 0.69 mg. og for mænd ca 0.86 mg. pr. døgn), hvorefter den igen falder i de *højere aldersklasser. Der er ret stor individuel variation i ud*skillelsen, men kun enkelte værdier (3 kvinder og 2 mænd) devierer mere end ± 50 % fra værdierne paa gennemsnitskurverne.

Beregnes udskillelsen pr kg. legemsvægt er den størst hos børn (ved 2 aars alderen ca. 14.5 μg. pr. kg) og mindst i de *højere aldersklasser (mellem 70 og 80 aar: ca 7.5 μg pr. kg).* Mellem 10 og 40. aar ændres den kun lidt. Forskellen i corticoidudskillelsen hos de to køn udjævnes betydeligt, naar udskillelsen udtrykkes paa denne maade. Indenfor samme aldersklasse synes der at være en vis proportionalitet mellem legemsvægt og corticoidudskillelse.

Beregnes udskillelsen pr. m² legemsoverflade formindskes aldersvariationerne betydeligt. Udtrykt paa denne maade er udskillelsen hos voksne indtil 50 aars alderen dog stadig lidt større end hos børn, medens den efter denne alder bliver lavere end hos disse.

De største daglige variationer i udskillelsen hos de tre kvinder (undersøgt gennem længere tid) var ca. ± 30 % af de enkeltes gennemsnitsværdier. Variationerne hos de 2 mænd, der *undersøgtes 14 og 7 dage, var noget mindre. Der fandtes ingen* relation mellem corticoidudskillelsen og menstruationscyklus hos kvinder

Resultaterne for 17-ketosteroid*udskillelsen adskiller sig* ikke fra dem, der er meddelt i litteraturen Det fremhæves, at disse steroider først fra 10—12 aars alderen udskilles i større mængder, og at saavel aldersvariationerne som kønsvariationerne i udskillelsen her er langt mere udtalt end for corti-

coiderne. Spredningen af enkeltværdierne er meget stor hos børn. Hos voksne er den noget større end for corticoiderne. Der synes ikke at være nogen relation mellem 17-ketosteroidudskillelsen og henholdsvis legemsvægt og -overflade.

Undersøgelserne viser, at corticoiderne og 17-ketosteroiderne udskilles uafhængigt af hinanden. Dette tages som et udtryk for, at de to steroidgrupper hidrører fra to af hinanden uafhængige funktioner af binyrebarken, der udvikles paa forskellige tidspunkter i livet.

Der synes at være en vis relation mellem celleaktiviteten (og dermed legemets energiomsætning) og corticoidudskillelsen (-produktionen). Formentlig er corticoiderne af afgørende betydning for visse intermediære stofskifteprocesser, men den nøjere mekanisme er ikke kendt. Disse forhold diskuteres indgaaende.

ERRATA

Part I: Page 74: HO = attached at C_{11} in 17-hydroxy-corticosterone, read: HO—

Part II: Page 226, line number 5 from beneath: by shaking 3 times, read: by shaking once.

Part III: Page 108, line number 2 from beneath, column 8: E. H. H. 0.72 mg of corticoids before ACTH, read: F. J. 0.72 mg etc

Part IV: Page 144, lowest line in case H. C., read: in case H. H.